オオムギ染色体の顕微画像のシミュレーション

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Computer Simulation for Microscopic Images of Barley Chromosomes

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Synopsis

Several microscopic images produced by three optical systems and stained by three dyes were first successfully simulated by the chromosome image analyzing system, CHIAS, using a single black and white phase contrast image.

Key words : Image simulation, Barley chromosomes, Phase contrast microscopy, Differential interference contrast microscopy, DAPI staining, Orcein staining, Giemsa staining.

Introduction

Several optical systems for microscopes have been developed during these decades. A phase contrast system, which enables to visualize the object without staining was developed by Zernike in the early 1930s (Gray 1973). Presently three different phase contrast systems are commercially available (Nikon Technical Bulletin 1981) depending on the experimental requirements. With a Dark-Low contrast (DL) system, transparent objects can be visualized and appear as light absorbing ones with low contrast. Dark-Medium contrast (DM) objective lenses give a higher contrast to images of transparent objects than a DL system. By using a Bright-Medium contrast (BM) optical system, or a negative phase contrast system which inverts the images, the object appears brighter than the background field. Differential interference contrast (D.I.C.) optical system which was devised by Nomarski in 1952 (Alberts *et at.*, 1983) enables to construct a microscopic image with a three dimensional appearance. These optical systems have been widely utilized in cytological and chromosomal studies.

Several staining methods have been also developed to facilitate chromosome

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observation. A basic dye, orcein that stains chromosomes dark red, was first employed as a chromosome stain by La Cour in 1941 (Sharma and Sharma 1980a) and is still one of the most popular dyes used in the plant chromosome research. The Giemsa stain is also widely used in chromosome studies both in plants and animals. The Giemsa stain which is a mixture of basic and acidic dyes (Sharma and Sharma 1980b), stains the chromosomes dark purple. A fluorescence dye, 4′-6-diamidino-2-phenylindole-dehydrochloride, DAPI that combines with chromosomal DNA, emits a whitish blue luminescence when it is illuminated by ultra-violet light (Lin and Alfi 1986).

All these optical chromosome images provide various aspects of chromosome characteristics and are now indispensable for chromosome studies. It had been, however, difficult to evaluate these images by ordinary methods. Therefore, we adopted imaging techniques to quantify the images. All the digital characteristics of the optical images were extracted and were programmed up to the command histories. Digitally generated simulation images were compared with the corresponding original optical images and the digital characterization was found to be adequate.

Materials and Methods

Plant materials and cytology.

Root tips of germinating barley seeds (*Hordeum vulgare* L. cv. 'Amagi Nijo') were excised and were pretreated in aerated distilled water at 0°C for 24 hr. After 15–30 min of fixation in 45% acetic acid, they were macerated for 30 sec. in 1N HCl at 60°C. Then

Table 1. Sequential procedures to obtain barley chromosome images with different optical systems and staining methods.

Step No.	Cytology	Types of objective lenses
1	Phase contrast microscopy (Dark-Low, DL)	Nikon Plan DL 100x Oil
2	Phase contrast microscopy (Dark-Medium, DM)	Nikon Plan DM 100x Oil
3	Phase contrast microscopy (Bright-Medium, DM)	Nikon Plan BM 100x Oil
4	Differential interference contrast microscopy (Nomarski type, D.I.C.)	Nikon Plan D.I.C. 100x Oil
5	Fluorescent dye staining (DAPI)	Nikon Plan-UVF 100x Oil
6	Basic dye staining (Orcein)	Nikon Plan-Apo 100x Oil
7	Mixed dye staining (Giemsa)	Nikon Plan-Apo 100x Oil

the root tips were cut into fine pieces with needles on a clean glass slide with 45% acetic acid. They were squashed after a cover slip was applied.

The barley chromosome spread was then examined subsequently by several optical microscopic systems and the staining procedures shown in Table 1. At first, a DL image of the chromosome spread mounted with 45% acetic acid was photographed through a microscope (Biophot, Nikon, Tokyo) at the magnification of 500x. All the microphotographs taken with the Biophot microscope provided the control images for the optical and staining manipulations of the images. Using the same metaphase spread, photographs of DM and BM images were taken at the same magnification. A D.I.C. image was also recorded by using a Nomarski optical system at the magnification of 625x. After taking four different optical images, the glass slide was dipped in liquid nitrogen and the cover slip was removed. The chromosome sample was dehydrated by dipping in a series of 70%, 100% ethanol and 100% xylene for five minutes each and air-dried.

The same glass slide was then dipped in 1μg/ml of a DAPI solution buffered with 2 M phosphate buffer (pH 7.0) for 1 hr. A photograph of the preparation with the DAPI stain was taken through a fluorescence microscope (Fluophot, Nikon, excitation wave length 365nm, absorption filter wave length 410 nm). The DAPI stain was washed with distilled water and the sample was completely air dried. Then a 10% aceto-orcein solution was dropped on the destained chromosomes, which were stained again in a moist chamber for 2 hr. Color microphotographs were taken by a Plan-Apochromat objective (100x, Nikon). Orcein was destained by dipping the glass slide in 45% acetic acid for 4 hr and dried. Then the slide was stained with the Giemsa solution (pH 6.8 1:150 dilution with a phosphate buffer) by dipping the glass slide in the solution for 30 min. After brief washing of the slide with water, it was air dried and the chromosome image was photographed. Color positive films (Kodachrome 64, ISO 64, Kodak, N.Y.) were used for light microscopy and high sensitive color negative films (Fuji Color HR1600, ISO 1600, Fuji Film Co., Ltd. Tokyo) for fluorescence photography.

Software and implementation.

Table 2 shows the outline for the simulation of microscopic images by the CHIAS (Fukui 1985, 1986, 1988). The programs for simulation of the microscopic images were constructed using the basic image manipulation commands provided by Zeiss/Kontron. The names of the digital filters were used according to the manual (IBAS 2 Users Manual 1984). Although the program required 176 commands altogether and was divided into four sub-parts, the main and essential digital image filters are only listed in the table. Opening program contains 13 commands and the total program length required for the command control is 134 bytes. It indicates the names of the programmer, the program contents, *etc.* The first main program for the simulation of the optical effects has 37 commands and the length for the command control is 386 bytes. The second main program for simulation of the differential interference contrast image has a 66 command sequence and requires 1020 bytes for its control. The

Table 2. Sequential digital procedures to simulate barley chromosome images obtained with different optical systems and staining methods.

Step No.	Implementation	
1	Image input	Zeiss Neofluar
	(Phase contrast microscopy)	100x Oil
2	Image enhancement	Normalization
	Pseudo-DM image	filter
3	Image inversion	Inversion
	Pseudo-BM image	filter
4	Generation of	Pseudo-plast
	Pseudo-3D image	filter
5	Color editing I	Look-up-table
	pseudo-DAPI staining	No. 406
6	Color editing II	Look-up-table
	pseudo-orcein staining	No. 410
7	Color editing III	Look-up-table
	pseudo-Giemsa staining	No. 409

fourth part of the main program which is constructed for the simulation of the stained images has 60 commands and 596 bytes for the control program. These additional commands besides the main commands listed in Table 2 contain a description of the title in each image, insertion of a small histogram of gray value distribution in the image, *etc.*

The objective lens (Neofluar 100x, Zeiss, Oberkochen, FRG) of Photomicroscope III (Zeiss) has a similar contrast to that of Nikon DL objectives. Thus the first image of the barley chromosomes was taken through a TV camera mounted on Photomicroscope III by using the objective lens. After analog/digital conversion, the image was digitally stored in an image frame memory (512x512 pixels with 8 bit gray level steps for each pixel) as the original digital image throughout the simulation. Photographs of the simulated digital images were taken using a color image recorder (CIR-100, Nippon Avionics Co., Ltd., Tokyo).

Results and Discussion

Plate 1a illustrates a digitized phase contrast image of barley chromosomes originally obtained by using a phase contrast optical system of Photomicroscope III and stored in the CHIAS. Plate 1b shows an original phase contrast (DL) image photographed through the Biophot microscope. Differences in the optical filters and the voltage of the lamps between the microscopes resulted in a slightly different color of the photographs. Except for differences in the detailed color tone, these two images were very similar to each other. Plate 1a was, thus, used as an original digital image

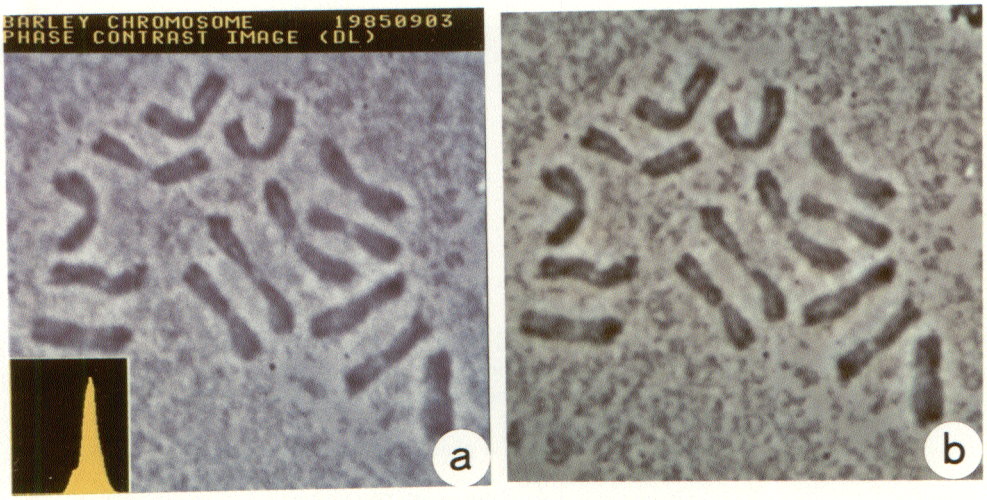


Plate 1. Original images for simulation.

a : Original digitized phase contrast image of barley chromosomes first obtained by using Photomicroscope III and digitally processed by the CHIAS. Histogram in the left lower corner indicates the distribution of the gray levels of the whole image. frame.

b : Original optical phase contrast (DL) image of barley chromosomes taken with a Biophot microscope.

Plate. 2. Simulation of three optical images.

a : Simulated digital image of a phase contrast (DM) image constructed from the digitized original image (Plate 1a).
b : Optical DM image.
c : Simulated image of a phase contrast (BM) image.
d : Optical BM image.
e : Simulated image of differential interference contrast (D.I.C.) image.
f : Optical D.I.C. image.

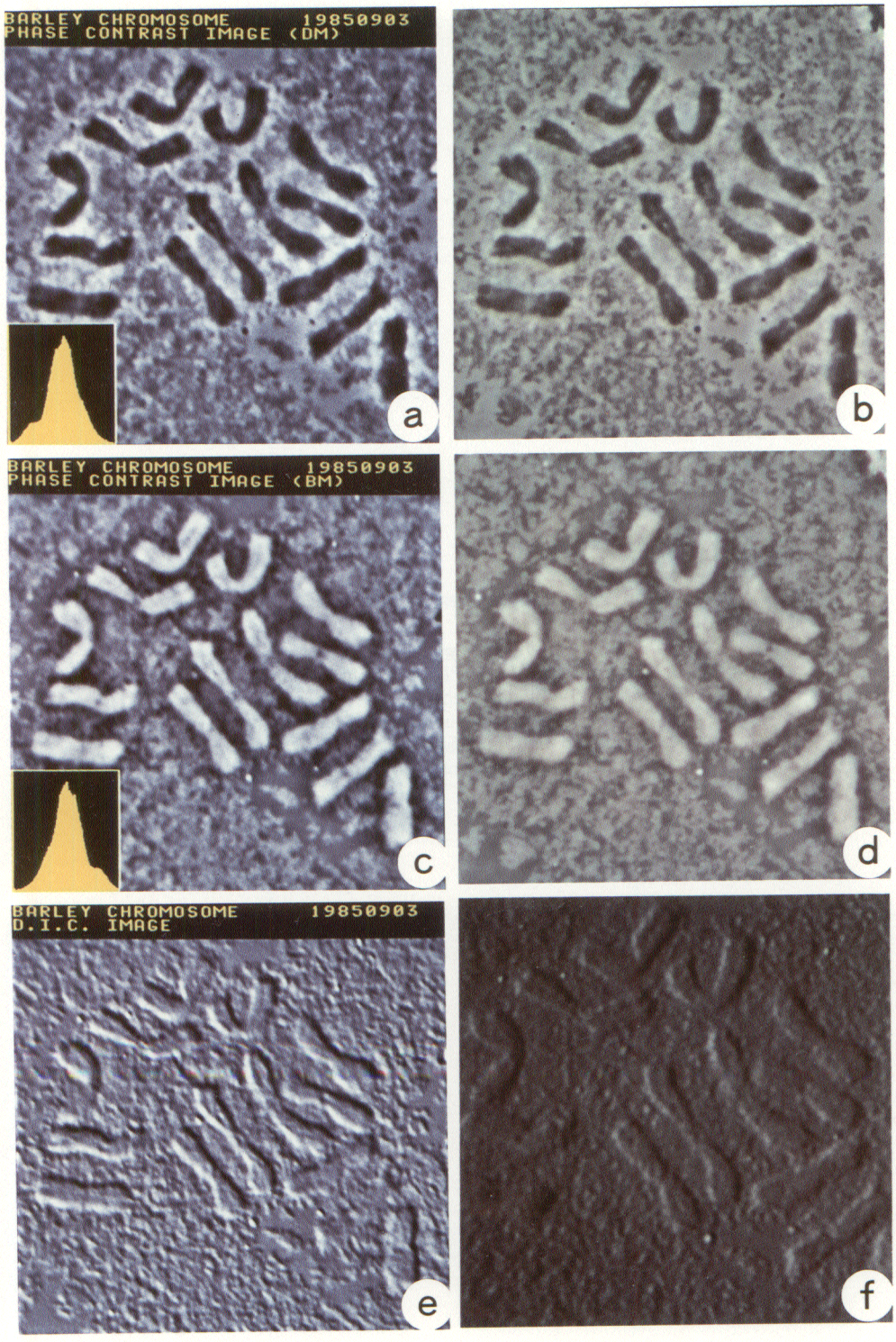
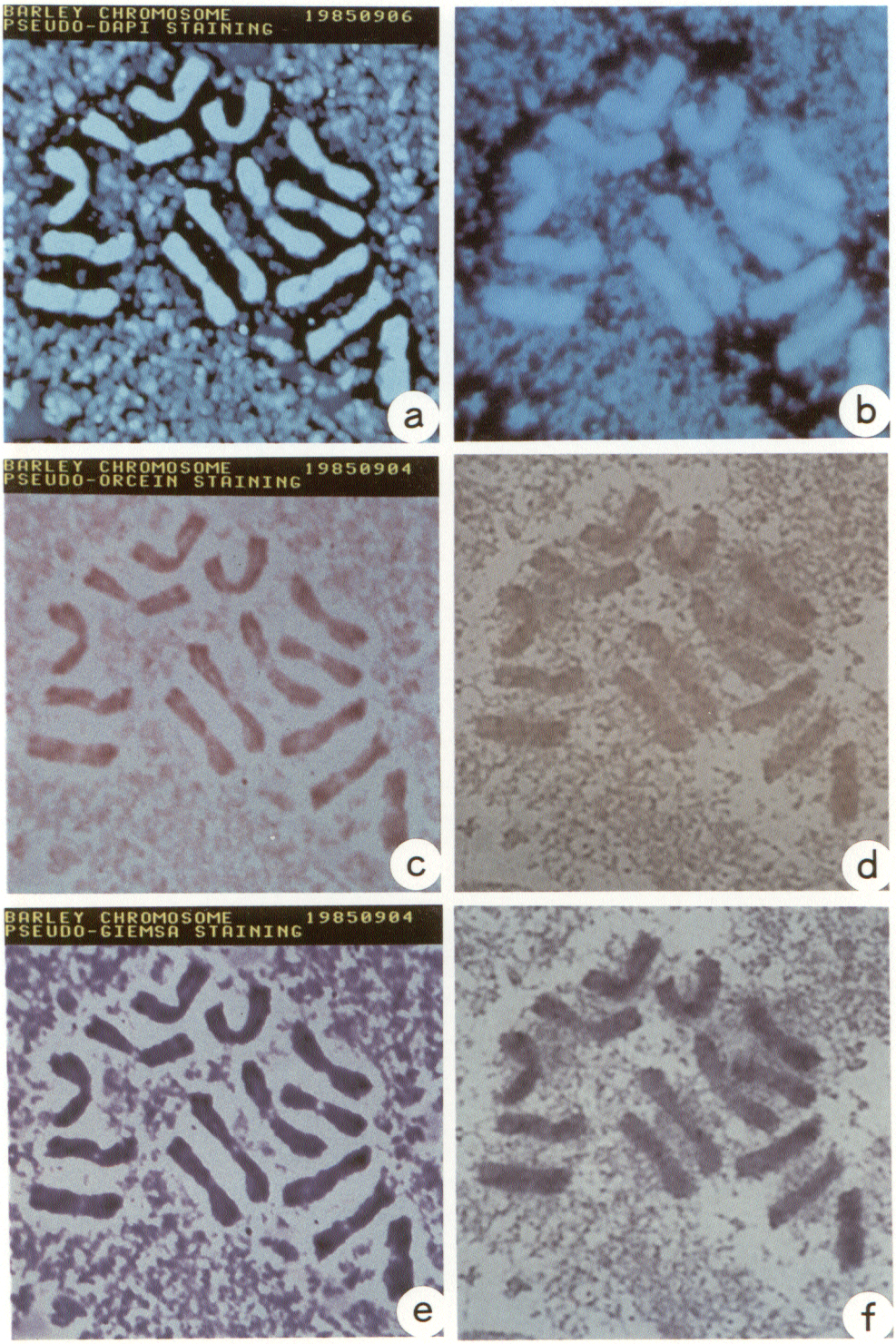

BARLEY CHROMOSOME 19850903
PHASE CONTRAST IMAGE (DM)
a
b
BARLEY CHROMOSOME 19850903
PHASE CONTRAST IMAGE (BM)
c
d
BARLEY CHROMOSOME 19850903
D.I.C. IMAGE
e
f

Plate 3. Simulation of three images of chromosomes stained with different dyes.

a : Simulated digital image of the DAPI-stained chromosomes.
b : Optical image of DAPI-stained chromosomes.
c : Simulation image of the orcein-stained chromosomes.
d : Optical image of orcein-stained chromosomes.
e : Simulated digital image of the Giemsa-stained chromosomes.
f : Optical image of Giemsa-stained chromosomes.


BARLEY CHROMOSOME 19850906
PSEUDO-DAPI STAINING
a
b
BARLEY CHROMOSOME 19850904
PSEUDO-ORCEIN STAINING
c
d
BARLEY CHROMOSOME 19850904
PSEUDO-GIEMSA STAINING
e
f

in order to simulate the optical and stained images throughout the study.

Plate 2 illustrates the results of the image simulation (Plate 2a, 2c, 2e) based on the original digital image (Plate 1a) by using the CHIAS and the corresponding optical images (Plate 2b, 2d, 2f) obtained by using the Biophot microscope. Plate 2b shows a DM phase contrast image. In the case of this optical system, the chromosome image appeared darker than the background field and the contrast of the image was enhanced compared to the original DL image (Plate 1b). In order to simulate the DM image that is characterized by these two points using a digitized DL image (Plate 1a), we compared and screened several digital image filters under various conditions mainly among the gray value transformation filters and we selected the 'Normalization' filter. This digital filter maps original gray levels linearly to the maximum range of 0 to 255. Histograms in the lower left corner of Plate 1a and 2a indicated the original and modified frequency distribution of the gray values by application of the filter, respectively. The horizontal axis of the histogram showed a gray value. Gray value 0, that is the darkest appeared at the left end and the right end showed the gray value 255, that is the brigthtest. The vertical axis indicated the frequency of the pixels. The histogram clearly illustrated the results of the image manipulation by the 'Normalization' filter and Plate 2a showed a clear resemblance to the optical DM image (Plate 2b).

Plate 2d shows the optical BM phase contrast image, which was characterized by a bright object compared with the background field with higher contrast than the original DL image (Plate 1b). Thus the original digital image (Plate 1a) was first subjected to the 'Normalization' filter treatment. Then the enhanced contrast image was subsequently processed by a digital filter, 'Inversion'. Algorism of the filter is;

$$\text{g.v.}(x,y) = \mid \text{o.g.v.}(x,y) - 255 \mid$$

where g.v.(x,y) shows the corresponding new gray value and o.g.v. (x,y) is the original gray value. By this operation for the gray value of each pixel, the frequency distribution of the gray values was converted to a distribution which was symmetrical to that of the original histogram for the gray value 127, as shown in the histogram in plate 2c. As a result, a newly constructed image could be simulated for the images obtained by the BM optical system (Plate 2d).

The image obtained by the D.I.C. optical system (Plate 2f) was simulated by using the CHIAS. As shown in Plate 3f, the image was relatively dark in the whole field and the chromosomes appeared in relief, that is in three dimensions with shade. Since Plate 3f was photographed at the highest contrast of the Nomarski system, the original digital DL image (Plate 1a) was first processed by the 'Normalization' filter in order to enhance the contrast of the chromosomes. Then, the high contrast digital DM image was subjected to the 'Pseudo-plast' filter treatment. Definition of the filter is as follows;

$$\text{g.v.}(x,y) = \left[\frac{1}{9}\sum_{k=-1}^{1}\sum_{\ell=-1}^{1}\text{o.g.v.}(x-k, y-\ell)\times h(k,\ell)\right] + 128$$

$$h = \begin{pmatrix} -1 & -1 & 0 \\ -1 & 0 & 1 \\ 0 & -1 & 1 \end{pmatrix}$$

where h is the size of a filter-matrix. As a result, the generated digital image (Plate 2e) appeated in relief like a three dimensional image illuminated from the left below.

Simulation of color images was carried out by using the 'Color Editor' function of the CHIAS. The 'Color Editor' generates different colors and allocates them to each pixel based on its original gray level. Each red, blue and green color has 256 steps of brightness. Thus more than 16 millions (256^3) different colors can be generated for each pixel by the combination of the three primary colors. A 'Look-up-table' whose function is a color arrangement according to defined programs implements the pseudo-coloration of gray images. 'Look-up-tables' suitable for DAPI, orcein and Giemsa stainig were constructed by programming several 'Look-up-tables' and by comparing the pseudo-color images generated by them with the color photograph of the original optical images shown in Plate 3. Firstly, the DAPI-stained image was simulated using the DL digital image (Plate 1a). In the case of the DAPI-stained image, chromosomal images with a smooth texture were observed usually as in the case of fluorescence dye staining (Plate 3b). Thus the smooth texture was simulated by the 'Median' filter at first. Its advantage lies in the fact that it does not blur edges and contours too much for larger objects like chromosomes as a lowpass filter application in this case. The 'Median' filter is a rank order operator and replaces all gray values of arbitrary size of the pixel matrix by the gray value of the central point on a ranking of gray level. As a result, the peripheral boundary lines of the chromosomes became slightly distinct and simulated the tones of fluorescence staining. Several 'Look-up-tables' were generated and tested to reproduce a DAPI stained image and 'Look-up-table' No. 406, whose numerical arrangement of three primary colors is listed in Table 3 was able to simulate the DAPI image shown in Plate 3a. Secondly, an orcein stained image was simulated. Orcein stains chromosomes dark red (Plate 3d). After generation of several 'Look-up-tables' and comparison among them, 'Look-up-table' No. 410 was selected as the most suitable one to simulate the orcein stained image using the original DL image (Plate 1a). Plate 3c illustrates the result of pseudo-orcein coloration. Finally, a Giemsa stained chromosomal image (Plate 3f) was simulated by the same method as DAPI and orcein staining. 'Look-up-table' No. 409 constructed a similar image to that obtained by the Giemsa staining (Plate 3e). These results show that it is possible to simulate the coloration with the staining solutions that stain chromosomes non-specifically, by the 'Color Editor' function of the CHIAS. Table 3 shows numerical corresponding data of gray values and the values of brightness for each primary color. Except for the 'Color Editor' function several other peripheral digital filters and

Table 3. Arrangement of three primary colors to gray values for color simulation of stained images.

	Gray value argument		Color data					
			Green		Blue		Red	
	From...To		IGV[1]	INCR[2]	IGV	INCR	IGV	INCR
Look-up-Table No. 406 (Pseudo-DAPI)								
1.	0	99	0	1	0	1	0	1
2.	100	125	250	−1	255	0	140	0
3.	126	145	230	0	255	0	140	0
4.	146	180	230	−4	255	−3	140	−5
5.	181	255	0	0	0	0	0	0
Look-up-Table No. 410 (Pseudo-orcein)								
1.	0	70	75	1	85	1	170	1
2.	71	100	145	0	155	0	230	0
3.	101	150	146	2	155	2	230	2
4.	151	255	240	0	240	0	240	0
Look-up-Table No. 409 (Pseudo-Giemsa)								
1.	0	90	0	1	0	1	170	1
2.	91	150	0	1	100	1	230	1
3.	151	180	60	4	180	0	230	3
4.	181	255	180	0	180	0	240	0

1) Initial gray value.
2) Increment of the gray value.

commands which modulated color tones and titled the images, *etc.* were used.

We demonstrated the possibility to simulate the efffect of the optical systems and staining colorations that are usually employed in cytological studies using only a black and white image, while securing complete reproducibility. Therefore, it was shown that the image analysis method provides an alternative means of utilizing the optical systems and staining procedures. It may be possible to construct a universal digital microscope which could generate all the different optical images without the use of equipment and any optical devices. By this successful achievement first obtained in the cytological field, the method described here could be utilized as a general method of the imaging technique since the digital filters employed for the simulation are now used by a large number of image analyzers. It is also anticipated that the image manipulation method may provide a completely new chromosomal image which may be very valuable for human visual characterization of the objects and also biologically significant.

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Summary

Barley chromosome images obtained by several optical systems of two phase contrast microscopy (Dark-Medium contrast, DM and Bright-Medium contrast, BM) and Nomarski differential interference contrast microscopy (D.I.C.) were simulated by the CHIAS using a black and white image obtained by phase contrast microscopy (Dark-Low contrast, DL) as the original reference. Color chromosome images stained with a fluorescent dye (4′-6-diamidino-2-phenylindole-dehydrochloride, DAPI), a basic dye (orcein) and a mixed dye (Giemsa) were also successfully simulated using the same original DL image by the CHIAS.

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和文摘要

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おしつぶし法で作成した1個のオオムギ染色体核板を無染色で位相差光学系およびDL，DMさらにはBM対物レンズを用いて観察し写真撮影を行った．ついでノマルスキー微分干渉光学系およびD.I.C.対物レンズを用いて染色体の半立体像を得た．同じ染色体核板について蛍光色素のDAPIを用いて染色体を染色し，蛍光顕微鏡により蛍光染色像を得た．DAPI脱染後，塩基性色素のオルセインで染色し，オルセインを脱染した後ギムザ液にて染色して3種類の染色像を得た．これらの7種の異なった光学像および染色像は染色体の種々の特徴を示しており，細胞学および染色体研究に不可欠なものとなっている．しかしながら従来の方法でこれら光学像を適切に評価することは困難であった．そこで画像解析法を用いてこれらの像を定量し，かつすべての画像の特徴を抽出し，プログラムの中にとりこんだ．このプログラムにより人為的に作出された画像と原画像とを比較することにより染色体の種々の特徴が適切にシミュレートされたことが明らかとなった．またこの方法により，各種染色体画像の種々の特徴を数量的に把握することが初めて可能となった．