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Color Image Analyzer and Its Application for Quantitative Morphology — Quantitative Fluorescence Image Analysis—

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So far quantitative fluorescence histochemistry has been carried out separately by two different methods, cytofluorometry for densitometry and image analysis for morphometry. We described here a method of **quantitative fluorescence image analysis** to realize simultaneous densitometric and morphometric measurements by using the color image analyzer. The summation of brightness values of the pixels in the frame buffer responded linearly and proportionally to fluorescence values determined by cytofluorometry, provided that brightness values were within an adequate range. This will enable us to extend quantitative investigation on histochemical reaction and shape analysis in variety of biological systems.

Introduction

With advantage of high sensitivity, fluorescence microscopy has been widely used as a histochemical and cytochemical method with the purpose of demonstrating a substance in very low concentrations (1,2,7,10,12). Moreover, the development of specific and stoichiometric fluorescence reactions enables us to perform cytofluorometric quantification of intracellular substances (3,5). Quantitative histochemical analysis by means of cytofluorometry, however, cannot give morphometric information of the cells or topological information on localization of an aimed substance within the cell.

Recently image analysis has become a dependable technique for morphometric or stereological quantitative analysis on histochemical and cytochemical reactions of various biological materials (4,8,9,11). Besides, it is, at least theoretically, possible in quantitative fluorescence reactions to determine the specific fluorescence intensity of a substance from the brightness of the pixels of image analyzer, even of a minute material below the resolution of a routine transmitted-light microscope due to high sensitivity of fluorescence microscopy(1,9). Although a few papers have reported fluorescence quantification by image analysis, the conditions that are necessary to perform an accurate densitometric analysis have not been investigated up to the present time.

The present paper describes the experiment for developing an analytical method of simultaneous precise densitometric and

morphometric measurements on a fluorescence image. Approach is to use color image analyzer (CIA) which has the capacity of cytofluorometry in addition to usual morphometric analysis. It is the method of **quantitative fluorescence image analysis.**

Materials and Methods

1.System Outline

Figure 1 shows a schematic diagram and actual photograph of our system for cytofluorometry and image analysis. The system consists of a cytofluorometer with an epi-illumination apparatus (Olympus, AH2-SRF), a silicon intensifier target (SIT) camera (Hamamatsu Photonics, C1000-12), a color image analyzer (Olympus, CIA) and a host computer (Hewlett-Packard, HP9520).

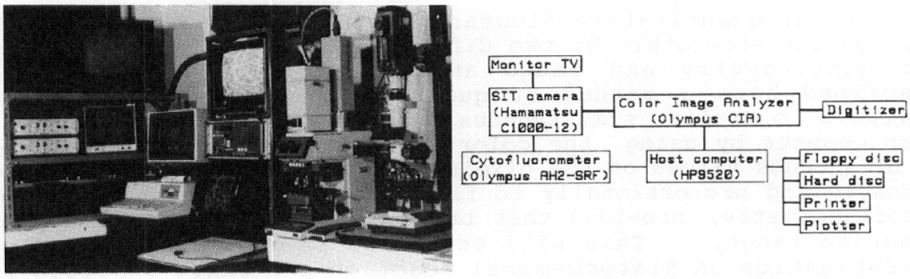

Figure 1. A combined system of cytofluorometer and fluorescence image analyzer

Microscopic fluorescence images were taken by SIT camera. The analog signals were digitalized by means of an 8-bit A/D converter, thus yielding up to 256 gray levels from 0 to 255. The data are stored in the frame memory which includes three separate 512x480x8 bits memory channels that are configured to correspond to the red, green and blue primary images of a normal color picture. In this study, we utilized a red memory channel for input of the image data and output of the results. Other memory boards were used for calculation and processing of the image data. The image in the RAM (Random Access Memory) was the digital representation of what the investigators wish to analyze and measure through the host computer with "macro-commands" (4). The cytofluorometer was also controlled under the host computer to offer increased flexibility and data-handling capacity.

2.Measurement Procedures

Figure 2 is a flow-chart diagram of the measurement procedures. The first step was to determine the DNA content by cytofluorometry after DAPI staining (6). In the second step, the fluorescence image was integrated from 10 to 1000 times by successive addition of 8-bit input images to a 16-bit resolution using two frame memory boards for removing random noise (so called pepper and salt noise) due to the dark current (Figure 3a and 3b). Adequate 8-bit image was selected from integrated 16-bit image. The third step was to obtain the density histogram

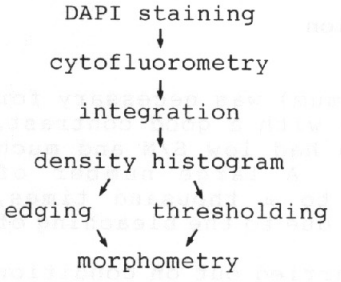

Figure 2. Flow-chart of measurement procedure

which revealed the number of pixels of given brightness in the cytofluorometry specific and background fluorescence image (Figure 3c). The next step was the extraction of the specific object image (Figure 3d). This was performed by two different techniques. One was edge enhancement by using filtering technique with 3x3. Another technique was thresholding of edge detection. The threshold level was determined as the largest differential value of brightness on scanning line. Because a steep ascending slope of brightness on scanning line indicates a border between specific figure and background on the image. The final step was measurements of morphometric parameters such as maximum length, maximum width, area, perimeter, etc. The densitometric informations were calculated from density histogram utilizing the light-transfer properties of video-camera.

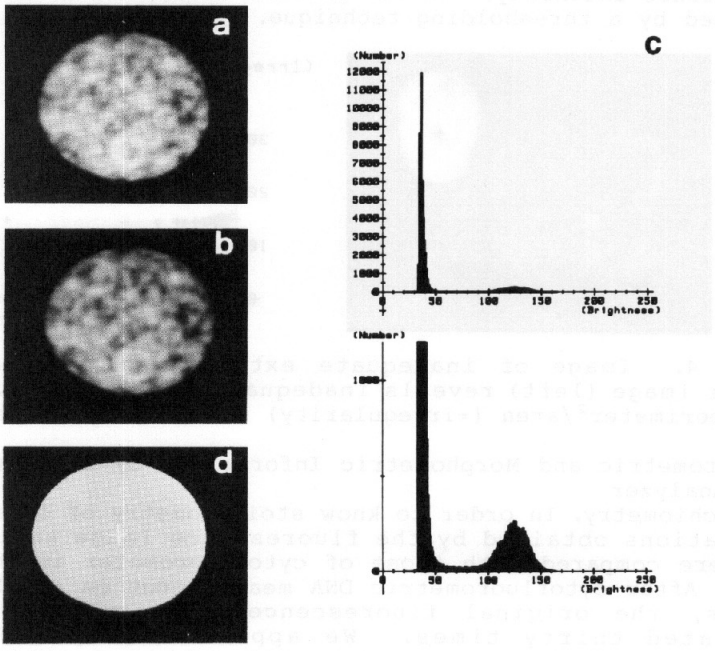

Figure 3. Image integration and extraction on a rat hepatocyte stained with DAPI. a:Original image before integration. b:Integrated image. Note the contrast without random noise. c:Density histogram of the image. X-axis indicates 256 gray levels from 0 to 255 and Y-axis, number of pixels. The first peak consists of the background pixels and the second peak, the specific pixels on nuclear area. d:Image extraction by thresholding technique, which is followed by morphometric analysis.

Results and Discussion

1.Integration and Extraction

Integration (thirty times, at minimum) was necessary for obtaining high signal/noise (S/N) image with a good contrast. The original image without integration had low S/N and much pepper and salt noise (Figure 3a). A large number of integration such as several hundreds to a thousand times, however, resulted in rather low S/N level due to the bleaching of specific fluorescence.

The complete extraction could be carried out on condition that fluorescence intensity which could be controlled with the iris of object lens was adequately adjusted to the dynamic range of SIT camera and A/D converter. When the range of fluorescence intensity was put to a lower level, the extraction became incomplete due to poor contrast. At the same time, the perimeter was elongated as shown in Figure 4.

The 3x3 space filtering by operator windows did not give good edging. Probably the reason is that biological materials such as cell nuclei possess wide and complex distribution of fluorescence intensity. In the present study, the extraction was performed by a thresholding technique.

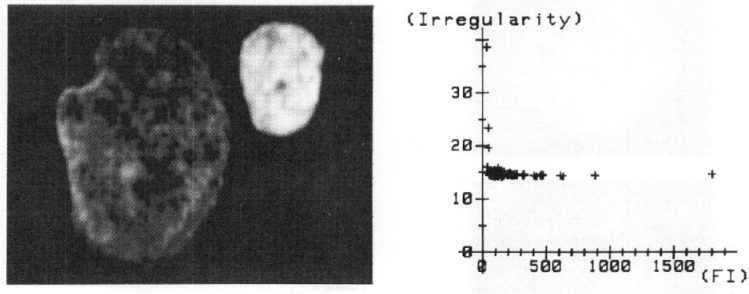

Figure 4. Image of inadequate extraction. Low-contrasted nuclear image (left) reveals inadequate thresholding. In this case, perimeter^2/area (=irregularity) becomes high (right).

2.Densitometric and Morphometric Information by the Fluorescence Image Analyzer

(i)Stoichiometry. In order to know stoichiometry of densitometric informations obtained by the fluorescence image analyzer, the data were compared with those of cytofluorometer in the present study. After cytofluorometric DNA measurement on a DAPI-stained nucleus, the original fluorescence image was successively integrated thirty times. We applied two parameters in measurement of fluorescence intensity, **B_total** and **B_specific** (Figure 5). The **B_total** represented a total of brightness of all pixels; $\sum_{i=0}^{255} Px(i) \cdot i$, where $Px(i)$ means a number of pixels with a brightness of i. Another parameter, **B_specific**, indicated a summation of specific brightness of pixels on nuclear area; $\sum_{i=bn}^{255} Px(i) \cdot i - Area \cdot bn$ (bn means a brightness of thresholding level

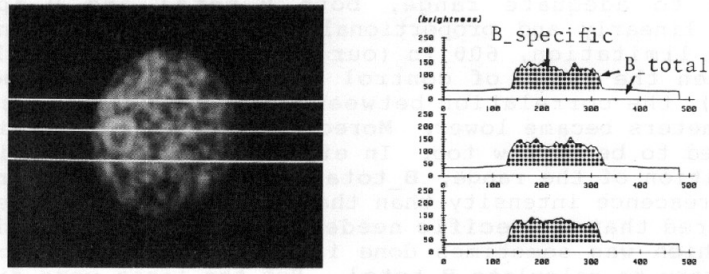

Figure 5. Brightness along three scanning lines. **B_total** represents a total of brightness of all pixels and **B_specific** indicates a summation of specific brightness of pixels limited on nuclear area.

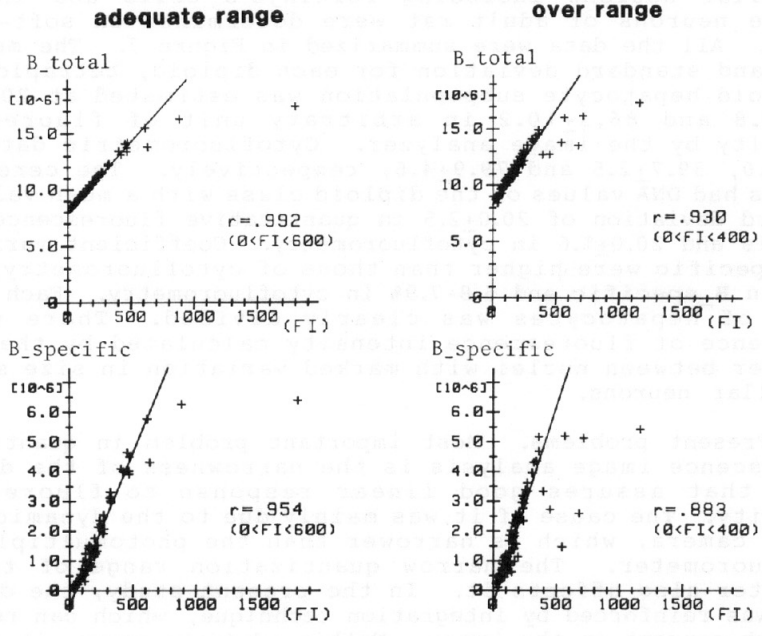

Figure 6. Relationship between fluorescence intensity (FI) measured by cytofluorometer and **B_total** or **B_specific** obtained by the image analyzer. Both parameters in the condition of adequate range are more precisely proportional to FI values than on the condition of over-range.

and *Area* means a number of pixels on nuclear area). In other words, **B_specific** represented a set of brightness gotten by subtracting background from **B_total.** These values were employed with an idea that the brightness of pixels responds linearly to fluorescence intensity. Because the camera and A/D converter in this study were characterized by linear response to light intensity.

The results are shown in Figure 6. On adjusting fluorescence

intensity to adequate range, both **B_total** and **B_specific** responded linearly and proportionally to fluorescence intensity under the limitation, 600 in (our device dependent) arbitrary unit. When the range of control switch rose by ten per cent (Figure 6), the correlation between fluorescence intensity and both parameters became lower. Moreover, the stoichiometric range was altered to be narrow too. In either on adequate condition or over-condition of the range, **B_total** revealed better correlation with fluorescence intensity than that of **B_specific.** The reason was inferred that **B_specific** needed the edging or thresholding process which was sometimes done incompletely. The process was not necessary to calculate **B_total.** But the lines were displaced vertically due to background in **B_total.**

(ii) Nuclear DNA measurements. By using the parameter **B_specific**, DNA ploidy patterns of the hepatocytes and the cerebellar neurons including Purkinje's cells and internal granule neurons of adult rat were determined on soft-touch-smears. All the data were summarized in Figure 7. The mean DNA value and standard deviation for each diploid, tetraploid and octaploid hepatocyte subpopulation was estimated at 20.0 ± 2.3, 41.7 ± 4.8 and 86.3 ± 10.2 in arbitrary unit of fluorescence intensity by the image analyzer. Cytofluorometric data were 20.0 ± 1.6, 39.7 ± 2.5 and 79.9 ± 4.6, respectively. The cerebellar neurons had DNA values of the diploid class with a mean value and standard deviation of 20.0 ± 2.5 in quantitative fluorescence image analysis and 20.0 ± 1.6 in cytofluorometry. Coefficient variations of **B_specific** were higher than those of cytofluorometry; 11.5-12.7% in **B_specific** and 5.8-7.9% in cytofluorometry. Each ploidy class of hepatocytes was clearly divided. There was no difference of fluorescence intensity calculated by the image analyzer between nuclei with marked variation in size such as cerebellar neurons.

(iii) Present problems. Most important problem in quantitative fluorescence image analysis is the narrowness of the dynamic range that assures good linear response to fluorescence intensity. The cause of it was mainly due to the dynamic range of SIT camera, which is narrower than the photomultiplier of cytofluorometer. The narrow quantization range of the A/D converter also affects it. In the present study, the dynamic range was reinforced by integration technique, which can restrain the dark current on the image. Within adequate range, it enabled us to measure the DAPI-DNA with good proportionality, even on cerebellar neurons which have marked regional differences in intensity of nuclear fluorescence. However, certain cell population with nuclear fluorescence much more heterogeneous such as cancer cells isolated from paraffin section might render a difficulty to carry out the DNA measurement with good stoichiometry.

The second problem is incomplete segmentation. As shown in Figure 4, segmentation by edging or thresholding was difficult especially in low contrast fluorescence images. High contrast images make easy to detect the edge or threshold border. Incomplete segmentation influences directly the stoichiometry of measurement values by the image analyzer.

Time consumption for image analysis occurred in a process of

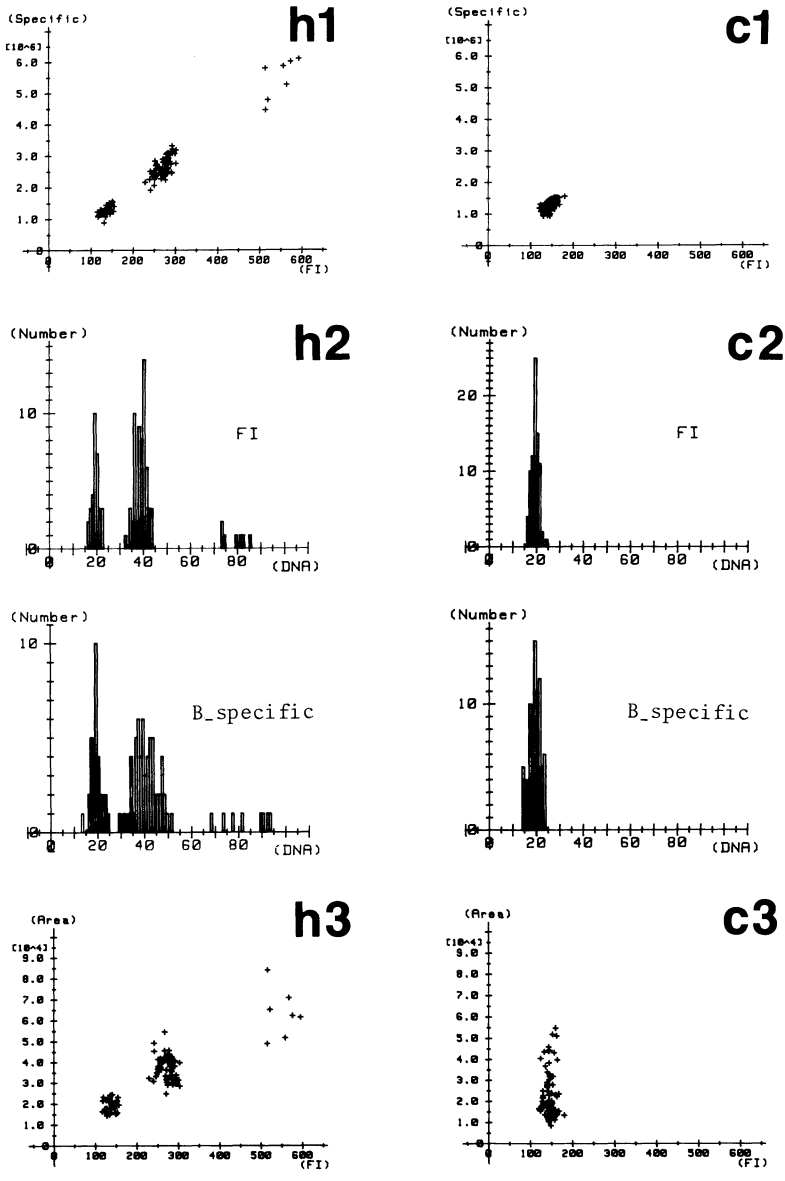

Figure 7. DNA contents of hepatocytes (h1) and cerebellar neurons (c1) determined by cytofluorometer (FI) and the image analyzer (**B_specific**). h2 and c2 show DNA-ploidy distributions, in which mean values of diploidy are adjusted to 20. h3 and c3 represent relationships between fluorometric DNA value (FI) and nuclear area (Area).

integration and calculation of the density histogram due to repeated "PUT" and "GET" with the "macro-commands" by BASIC via an HP-IB interface. These processes will be less time-consumptive if the commands of routine work are written in Read-Only-Memory (ROM) and placed in the image analyzer.

Conclusion

More complete histochemical analysis needs a method of simultaneous densitometric and morphometric measurements. Although the exact density information is dependent on cytofluorometric technique at present, it is clarified in this paper that not only morphometric informations but also densitometric informations can be obtained by **quantitative fluorescence image analysis**, provided that the measurements are carried out within an adequate dynamic range.

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