Microphotometry of banded human chromosomes

High resolving power by direct scanning of the specimen compared with scanning on microphotographs

C. LUNDSTEEN and J. PHILIP

Chromosome Laboratory, Department of Obstetrics and Gynaecology Y, Rigshospitalet, University of Copenhagen, Denmark

Summary. A method which was designed for microphotometric measurements of banded human chromosomes using a computer-controlled Zeiss scanning microscope photometer (SMP) is described. Modification of the Apamos 2 program (H. G. Zimmer, Zeiss, Oberkochen) enabled the scanning stage on the SMP to move the slides continuously thus obtaining high resolution of measurements. Results of scanning directly on the slides when compared with scanning on microphotographs revealed no significant differences; the banding patterns of the chromosomes were reproduced with a high degree of accuracy by both methods. Chromosomes No. 2, 10, 16, and 20 are described in detail.

In the past few years methods have been described for densitometric measurements on microphotographs of banded human chromosomes (Caspersson, Lomakka, and Møller, 1971; Caspersson *et al.*, 1971; Fleischmann *et al.*, 1972; Møller *et al.*, 1972); in this way objective and exact registration of banding patterns can be obtained. This is a condition for automatic identification by computer analysis of each chromosome. In a recent paper, Lundsteen, Ernst, and Philip (1973) described a method for measuring the banding patterns directly on the slide thus avoiding the photographic stage. The method has now been improved in several aspects. It is the purpose of this paper to describe the improvements and to give the results of comparing direct scanning on slides with direct scanning on photographs.

Materials and Methods

Human metaphase chromosomes were prepared according to a modification of the method of Moorhead *et al.* (1960) and stained with trypsin-Leishman (TL) (Seabright, 1971). Selected metaphase figures were also photographed (magnification $\times 400$) and the negatives mounted on slides without embedding medium and fixed with adhesive tape.

Received 24 August 1973.

This work was supported by the Danish Medical Research Council.

Straight chromosomes with distinct banding patterns were selected and scanned directly on the slide and on the negatives using the SMP 05 (Zeiss), under computer control (PDP 12 A). The Apamos 2 program (H. G. Zimmer, Zeiss) was modified for this purpose.

The chromosomes were orientated on the scanning stage so that the scanning took place parallel to the longitudinal direction of the chromosomes. The scanning stage on the SMP usually moves stepwise by means of steppermotors at a speed of 50 steps per second. Having completed one scan-line it makes one step in the vertical direction before the next line is scanned. The steppermotors may be controlled by computer in order to move the stage one step forward and stop for a short time before the next step. For the present purpose the program was changed so that the number of impulses from the computer was increased to 200 per second. The motor therefore received the next impulse while still moving and the stage therefore moved continuously along the scan-line.

For direct scanning on the specimen a stage with a minimal stepsize of $0.5\ \mu\text{m}$ was used, thus 200 'continuous steps' of $0.5\ \mu\text{m}$ were performed per second. Four photometric measurements were made for each step of $0.5\ \mu\text{m}$ along a scan-line; that is one measurement per $0.125\ \mu\text{m}$ shift. Thus 80 measurements are made in scanning one length of a $10\text{-}\mu\text{m}$ long chromosome. A circular measuring diaphragm with a diameter of $0.5\ \text{mm}$ was used corresponding to $0.5\ \mu\text{m}$ on the specimen. In the vertical direction the stage made steps of $0.5\ \mu\text{m}$.

For scanning on negatives a $10\ \mu\text{m}$ stage was used.

Fig. 1

Figs. 1 and 2. Line a: printout of scanning of the microphotograph; line b: direct scanning of the specimen; line c: microphotograph of the chromosome. Dark bands are numbered according to the nomenclature suggested by the Paris Conference (1971). FIG. 1 shows homologous No. 2 chromosomes. FIG. 2 (on p. 198) shows chromosome No. 10 on the left; No. 16 in the centre, and No. 20 on the right

The stage moved with 200 'continuous steps' of 10 μm per second. One photometric measurement was performed per 30 μm movement, therefore in scanning one length of a 10- μm long chromosome (corresponding to 4000 μm on the negative) 130 measurements are made. The diameter of the measuring diaphragm was 1.25 mm corresponding to 30 μm on the negative, that is about 0.08 μm on the specimen. Between each scanline a vertical step of 30 μm was made.

All results were stored on tape on the PDP 12 A computer. The printouts are computerized calculations (modified Apamos 2) of the light absorbance or density of each point along the longitudinal direction of the chromosomes. The calculations give the sequence of dark and light bands and their size and staining intensity. Thus the printout diagrams represent the banding pattern of the scanned chromosomes.

Results

In Figs. 1 and 2, line a is a printout of the scanning on the negative, line b is the direct scanning on the slide, and line c is a microphotograph of the chro-

mosome. The scale is identical for diagrams and photographs in the longitudinal direction of the chromosome.

Vertical lines are drawn to indicate the dark bands seen on the microphotographs and the corresponding spikes on the diagrams. They are numbered according to the nomenclature suggested by the Paris Conference (1971). All unambiguous bands are marked and the centromeres are indicated by a continuous line.

Figure 1 illustrates two homologous No. 2 chromosomes. On both chromosomes 11 dark bands are identified, some darker and more distinct than others. On the left-hand chromosome bands p24, p22, p16, p12, q14, q22, q32, and q34 are rather distinct on the photograph and can be easily recognized as spikes on both lines a and b; the faint bands q12 and q36 are not as pronounced on the diagrams, and q24 is not clearly separated from q22 either on the photograph or on the diagrams. On the right-hand chromosome bands p24, p12, q22, q32, and

FIG. 2

q34 can be easily identified on both photograph and diagrams; whereas the very faint bands q12 and q14—as well as the curved band q36—can be only identified with difficulty on the diagrams. p16 and p22 are badly separated on the photograph and diagrams, though somewhat more distinctly in line a than line b.

Figure 2 illustrates chromosomes No. 10 (left), No. 16 (centre), and No. 20 (right). On chromosome No. 10 all five dark bands are distinct and clearly separated, and can be identified on the two diagrams as distinct spikes. On chromosome No. 16 it can be seen that the faint band p12 is clearer on the diagrams than is band q23, which is not a straight line. On chromosome No. 20 all three bands seen on the photograph—including even the very faint band q11, 2—can be recognized on the diagrams.

Discussion

The method described represents considerable progress compared to the method using the UMSP (Zeiss) used in earlier experiments (Lundsteen *et al*, 1973).

The SMP is more easily adjusted mainly because it is a single beam instrument. The scanning stage is easily moved in the longitudinal and vertical direction by a joystick in order to position the chromosome correctly, and the scanned area can be controlled by a special eyepiece. A special object holder is under construction so that the slide may be rotated without losing the metaphase from the field of view.

The speed of the whole scanning procedure is important. If the method is to have practical value it must not be too time-consuming. In the experiments performed the scanning procedure itself took only 1 to 2 seconds, while adjusting the instrument and correctly placing the chromosome required between 2 and 10 minutes. Storing the results in the computer took about 1 to 2 minutes. We believe that by further improvements of the method one chromosome may be selected, scanned, and the results stored within 2 to 3 minutes. This means that one metaphase may be completed in about 2 hours. The SMP is much faster than the UMSP mainly because the results of the measurements are directly transferred to the computer; storage on punch tape is not necessary.

High resolving power was reached by increasing the number of photometric measurements along a scan-line in such a way that one measurement was performed for each 0.125- μ m movement of the stage. This was possible only because the stage moved continuously. As the diameter of the

measuring diaphragm was 0.5 mm, corresponding to 0.5 μ m on the specimen, the measuring areas overlapped each other. A 0.5-mm diaphragm was chosen mainly because the minimal stepsize in the vertical direction was 0.5 μ m. Smaller diaphragms may be used in order to reduce the overlapping, but this will increase the diffraction due to the diaphragm. However, the overlapping of the measuring areas seems less important as the primary aim is to determine the location of stain maxima and minima (Lundsteen *et al*, 1973), and these determinations are independent of the overlapping.

The results show that all distinct bands seen on the microphotographs could be identified on the printouts both from the direct scanning and the scanning on photographs; very faint and curved bands were not quite as well illustrated. As faint bands may be more variable and difficult to reproduce, and artefacts may influence faint bands more than distinct ones, the value of measuring these bands seems questionable.

Only small differences were found between the results obtained after direct scanning and after scanning on microphotographs. With both methods distinct and well-separated bands on the chromosomes were clearly reproduced as spikes, while very faint bands and bands lying very close to each other seemed slightly better pronounced after scanning on photographs. The importance of these small differences is questionable, but the problem will be further investigated.

The method did not allow registration of the minor band variations in staining intensity across the chromosome because the computer program was designed only for registration of the staining intensities along the chromosome and because high resolving power was only obtained along the horizontal scan-lines. In the vertical direction the stage moved in steps of 0.5 μ m. The resolving power in the vertical direction could perhaps be improved by mounting a vibrating mirror in front of the photomultiplier; it is intended to build such a mirror into the microscope. By performing four times as many photometric measurements as before, the same high resolving power should be reached in both longitudinal and vertical direction. With improvement of the software information for centromere position, chromosome area, total and relative light absorbance, and other values of interest may also be obtained.

The possible applications of the method have been previously discussed (Lundsteen *et al*, 1973). If the large amount of information contained on banded chromosomes is to be made useful it will be necessary to quantitate and computerize this

information. By extracting the characteristic features from the stored data it may be possible to classify each chromosome by special computer programs (Møller *et al*, 1972). It may be possible to determine whether differences exist between the banding patterns of chromosomes from different individuals and between homologous chromosomes; chromosome rearrangements might be exactly identified and minor structural abnormalities might be diagnosed and perhaps related to abnormal phenotypes. The method could be of great value in comparative studies of different banding techniques. At this time the method is considered only to be a semi-automatic system not meant for routine use; ultimately complete automatic chromosome identification may be obtained.

From our experience to date we would conclude: (1) that by photometric scanning, objective and exact information regarding position, size, and staining intensity of all distinct bands may be obtained; (2) that the resolving power of the system seems sufficient; (3) that no significant differences have yet been found between scanning of slides and photographs; (4) that further studies must be carried out for indistinct bands; (5) that compared to the UMSP, the SMP + PDP 12 represents an improved system; as the SMP is computer controlled, the chromosomes are scanned much faster and the

special object holder facilitates the adjustment of the chromosomes.

We are most grateful to the Zeiss Foundation for allowing us to carry out the measurements in the laboratories in Oberkochen. We wish to thank Dr H. G. Zimmer for important technical advice, for providing the special computer programs, and for kindly reading the manuscript. We also wish to thank Mr H. Wintersteiner for valuable technical assistance.

REFERENCES

- Caspersson, T., Castleman, K. R., Lomakka, G., Modest, E. J., Møller, A., Nathan, R., Wall, R. J., and Zech, L. (1971). Automatic karyotyping of quinacrine mustard stained human chromosomes. *Experimental Cell Research*, **67**, 233-235.
- Caspersson, T., Lomakka, G., and Møller, A. (1971). Computerized chromosome identification by aid of the quinacrine mustard fluorescence technique. *Hereditas, Genetiskt Arkiv*, **67**, 103-110.
- Fleischmann, T., Gustafsson, T., Hakansson, C. H., and Levan, A. (1972). The fluorescent pattern of normal chromosomes in biopsies of malignant lymphomas, and its computer display. *Hereditas, Genetiskt Arkiv*, **70**, 75-88.
- Lundsteen, C., Ernst, P., and Philip, J. (1973). Microspectrophotometry of trypsin-Leishman-stained human chromosomes. *Nature*, **242**, 613-615.
- Møller, A., Nilsson, H., Caspersson, T., and Lomakka, G. (1972). Identification of human chromosome regions by aid of computerized pattern analysis. *Experimental Cell Research*, **70**, 475-478.
- Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., and Hungerford, D. A. (1960). Chromosome preparations of leucocytes cultured from human peripheral blood. *Experimental Cell Research*, **20**, 613-616.
- Paris Conference (1971). Standardization in human cytogenetics. *Birth Defects: Original Article Series*, **8**, pt. 7, 1972. The National Foundation—March of Dimes, New York.
- Seabright, M. (1971). A rapid banding technique for human chromosomes. *Lancet*, **2**, 971-972.