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INFLUENCE OF MICROSCOPICAL MAGNIFICATION ON SPEED AND QUALITY OF DNA MEASUREMENT OF ARCHIVAL TUMORS

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ABSTRACT

The aim of this paper is to access to the influence of microscopical magnification on image cytometry DNA ploidy measurements, focusing especially on performances of automatic sorting of objects and time consuming. Results obtained show that performances of an automatic sorting method can vary to a large extent according to the chosen microscopical magnification.

Key words : DNA ploidy measurements, microscopical magnification, automatic cell sorting

INTRODUCTION

Estimation of DNA content of archival tumors represents an important challenge for the estimation of tumor prognosis. Recent works showed that densitometric measurement by image analysis (ICM) is the best way to get this information, due to the facilities of cell sorting (Herlin and Duigou, 1997). Nevertheless, the implementation of this technique in clinical oncology asks for full automation of the process, in order to measure DNA content of a statistically representative population (more than 750 nuclei) and to quickly collect reproducible information, while preserving precision.
Precision of the estimation of DNA content depends on the quality of image acquisition, image segmentation, and elimination of debris and aggregates generated during sample preparation (Boudry, 1997). These unwanted elements can be separated in four classes: small debris, sliced nuclei, damaged nuclei and aggregates and must be precisely removed from DNA ploidy measurements (Boudry et al., 1997). The two later categories can be distinguished from undamaged nuclei, which are mainly convex, by quantifying concavity on binary images.
The visual appreciation of concavity on digitised images depends on the chosen magnification. It seems likely that automatic measurement of this parameter is influenced in

the same way. Modifying the magnification ends also in a modification of the incident light intensity collected by the camera. This modification is able to influence the quality of segmentation of image and consequently the relative size of objects to sort.
The influence of microscopical magnification on the quality of measurements in biology has been scarcely explored until now (Belien et al., 1997).
The present paper discuss the influence of the chosen microscopical magnification on automatic characterisation of damaged nuclei and aggregates, in terms of performances and time consuming, using mathematical morphology based on automatic sorting method.

MATERIAL AND INSTRUMENTATION

Biological material

The study was performed on a case of archival brain tumour (astrocytoma grade 2) prepared according to Van-Driel Kulker et al. (1987) and Duigou et al. (1997).

Image acquisition

The acquisition device consists of a BH2 Olympus microscope, a scanning stage, a Matrox PIP 1024 frame grabber, and a Sony CCD camera. Acquisition is driven by a P.C. (Hewlett Packard) running under Unix operating system.
Acquisition was done at two magnifications: final magnification x 125 (objective 20, N.A.=0.7, additional lens x6.25) and final magnification x250 (objective 40, N.A. =0.85, additional lens x6.25). The size of images was 512x512 pixels. One hundred images were stored: fifty at a microspical magnification of 125 (low magnification) and fifty at a microspical magnification of 250 (high magnification).
In order to limit the decrease of the incident light intensity at high magnification, lamp voltage was raised in order to reach the proper level of video output signal of the C.C.D. camera (which is fixed). The validity of the correction of the incident light intensity has been controlled on the mean grey level histogram of the fifty images obtained at each magnification.
In order to evaluate the performances of automatic characterisation of damaged nuclei and aggregates, strictly on the same objects, the same fields were acquired using the two magnifications. Objects situated only in the middle of the low magnification images must be taken into account. To get strictly the same field of analysis for the two magnifications, low magnification images were cropped (256x256 pixels from x and y equal to 128) (Figure 1).

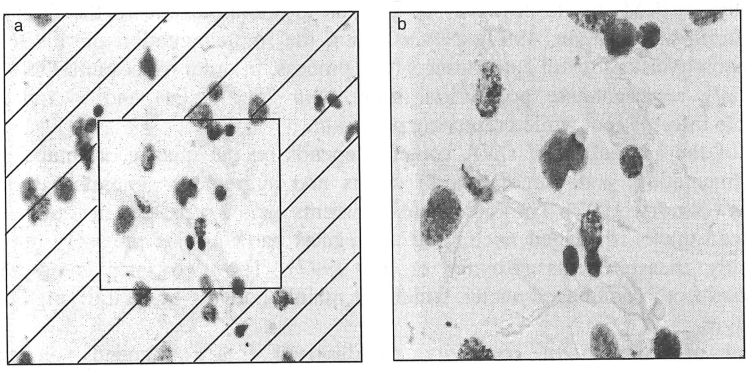

Fig. 1: Low magnification image (a) with a measurement frame corresponding to high magnification image (b).

The main disadvantage of this protocol is the inaccuracy of the position of the optical centers of the two objectives. It requires a visual control to verify that the objects are located exactly at the same location at low and high.

Segmentation method
Binary images were obtained thanks to an automatic threshold method based on image grey level histogram analysis (maximisation of interclass variance according to Otsu (1979)). To investigate the influence of the magnification on the quality and quantity of segmented objects, the surface area and the number of objects were evaluated for the two magnifications.

Sorting method tested
The maxima of dodecagonal distance function are classically used to characterise concavity (Serra, 1982, Coster and Chermant, 1989, Schmitt and Mattioli, 1993), keeping in mind that dodecagonal distance function of convex objects has one minimum, whereas concave objects have two or more maxima. This approach proved to be unable to discriminate slightly concave undamaged nuclei from damaged nuclei or aggregates. Computation of Rmin/Rmax ratio (Grimaud, 1991) as then been introduced as it allows a better quantification of concavity (Boudry et al. 1996). Rmin corresponds to the size of the neck between convex parts of objects and is computed thanks to a watershed transformation from the inverted image of dodecagonal distance with its minima as markers. Rmax corresponds to the largest part of objects (equal to the maximum of the dodecagonal distance function). Convex undamaged nuclei having only one maximum for dodecagonal distance function, their Rmin does not exist (Figure 2a). On the contrary, concave undamaged nuclei must exhibit high Rmin/Rmax ratio (Figure 2b), whereas damaged nuclei or aggregates must have low ones (Figure 2c and 2d). A simple Rmin/Rmax ratio threshold (giving the best contrast between undamaged nuclei and damaged nuclei and aggregates) has been performed to separate undamaged nuclei to be analysed from unwanted elements. Automatic sorting procedure method has been developed using Visilog 4.1.4 software routines.

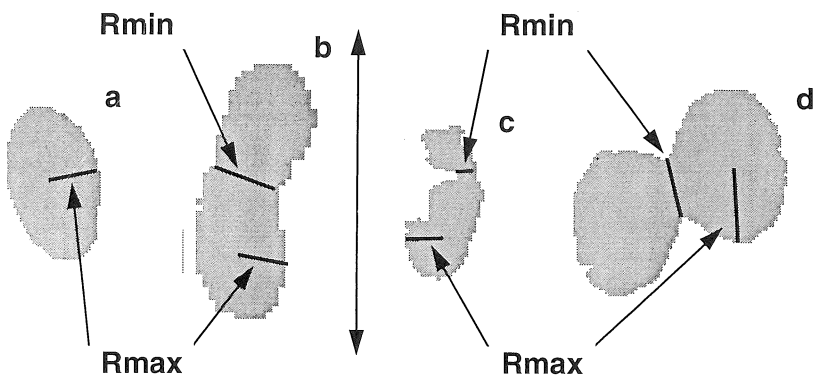

Fig. 2: Notion of Rmin/Rmax ratio: (a) for one concave undamaged nucleus (lack of Rmin), (b) for one concave undamaged nucleus, (c) for one damaged nuclei and (d) for one aggregate

Comparison of performances of automatic sorting method at high and low magnification
Segmented objects, corresponding to 244 elements were interactively classified as undamaged

nuclei on one hand, damaged nuclei and aggregates on the other hand, assuming that this interactive sorting corresponds to the reference sorting. For each microspical magnification, the performance of automatic method was assessed thanks to the computation of sensitivity (S) and false positive rate (FP). S is defined as the percentage of damaged nuclei or aggregates, correctly classified by automatic method, by reference to interactive sorting. FP is defined as the percentage of undamaged nuclei misclassified as damaged nuclei or aggregates. The adaptation of automatic sorting method from low magnification to high magnification, was simply obtained by increasing twofold the size of the structuring elements used.

RESULTS

Characteristic of images at low and high magnification

Despite correction of the incident light intensity, images acquired at high magnification were darkest than those acquire at low magnification, as shown by a shift of 23 grey levels on the histogram (Figure 3).

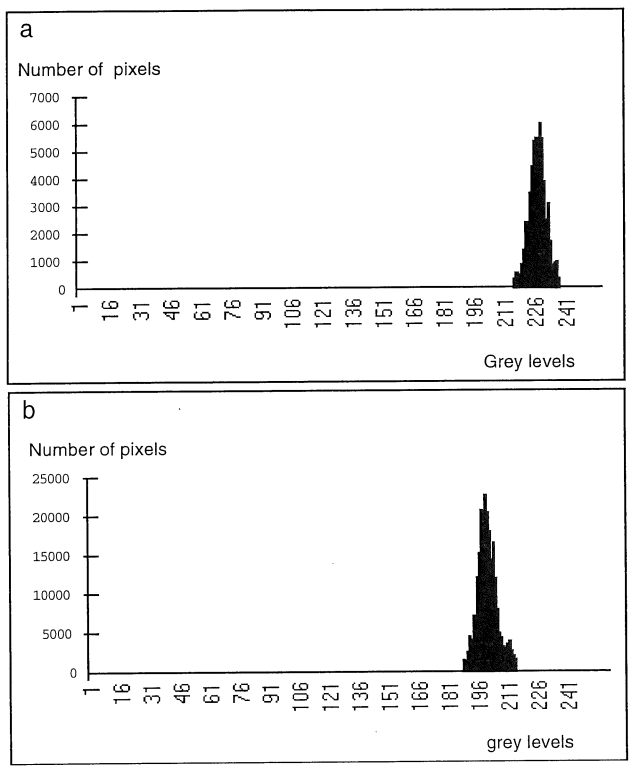

Fig. 3: Mean grey level histogram for the fifty images studied. (a) low magnification, (b) high magnification.

However, one must notice that the standard deviation of grey level values corrected by the mean was strictly the same (0.136) at low and high magnification.

Quantitative and qualitative performances of segmentation procedure
The segmentation according to the method of Otsu (1979) gives an equivalent number of objects at low and high magnification (244). Furthermore, the ratio of surface area obtained at high and low magnification was equal to 3.97 as compared to the theoretical 4 expected value, which represents an error of 0.99%.

Performances of the automatic method at low and high microscopical magnification
Table I gives the performances of the automatic sorting method to characterise damaged nuclei and aggregates at the two magnifications.

Table I: Performances of the automatic sorting method

	Low (×125)	High (×250)
S (Damaged nuclei) (n=65)	38.5	56.9
S (Aggregates) (n=44)	86.3	91
FP (Undamaged nuclei) (n=135)	1.4	9.6

Even if the twofold increase of the magnification increases FP (from 1.4 % to 9.6%), it increases at the same time the percentage of unwanted elements characterised (from 38.5% to 56,9% for damaged nuclei and from 86.3% to 91% for aggregates), which represents an increase of performances of 32.3% and 7% respectively.

DISCUSSION

Images obtained at high magnification were darker compared to those obtained at low magnification: it seems to be logical because the modification of the microscopical magnification leads to use two different objectives with different transfer functions. Despite the correction performed on the incident light intensity for the acquisition of high magnification images, a shift of 23 grey levels had persist. This may be due to the imprecision of this correction which was only guided by the vision of the proper level video output signal of the C.C.D. camera. Nevertheless, statistical characteristic of images obtained were similar.
It must be noticed that one way to avoid the problem to use two different objectives is to acquire images at high magnification and to generate low magnification images, keeping in the image only one pixel out of two. If this solution is attractive, we did not choose it as it did not reflect the reality of experimentation !
In order to avoid the influence on the performances of the automatic sorting method at the two magnifications tested, segmentation method used must strictly lead to the selection of the same objects, from a quantitative and qualitative point of view. Segmented objects obtained at low and high magnifications must be the same and must have an equivalent surface area. It was the case using the Otsu algorithm, which is based on image grey level histogram analysis. Nevertheless, other segmentation methods may not have fulfilled these conditions.
Performances of automatic sorting method are higher for undamaged nuclei than for aggregates when using high magnification. One must notice that most of the aggregates has enough marked concavities to be characterised at low magnification and in these conditions the increase of the magnification does not increase significantly their characterisation. On the contrary, most of damaged nuclei has not enough marked concavities to be characterised at

low magnification and the increase of the magnification increases significantly their characterisation.

CONCLUSION

Clinical estimation of DNA content of archival tumors must fulfil two conditions: precision and rapidity of measurements. Rapidity requires the complete automation of each step while precision asks for a precise control of every parameters that can influence DNA ploidy measurements. Microscopical magnification has proved to belong to this last category and performances of cell characterisation are higher at high magnification.
Nevertheless, one must keep in mind that the exploration of objects at higher magnification requires a greater number of images to analyse (to a squared factor) and consequently is more time consuming, so a compromise must be chosen.

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