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VOLUME DETERMINATION OF HUMAN METAPHASE CHROMOSOMES BY SCANNING FORCE MICROSCOPY

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Abstract

The scanning force microscope (SFM) yields the topography of the investigated surface. A procedure was developed which starts from this three-dimensional information to estimate the volume of a biological specimen. The volume of spread human metaphase chromosomes was determined in air and rehydrated in aqueous buffer. A difference of the determined volume of a air-dried metaphase chromosome set was found compared to values from electron microscopic investigations, and could be correlated with differences in the hydration state of the chromosomes. SFM-based relative volumes of air-dried chromosomes resembles literature data regarding volume range and distribution. Possible application of SFM-based relative volume measurements for chromosome classification purposes is discussed.

Key Words: Atomic force microscopy, chromatin, chromosome classification, karyotype, volume investigation.

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Introduction

Scanning force microscopy (SFM) results in the three-dimensional topography of the investigated surface. In contrast to transmission electron microscopy, which gives a two-dimensional projection of the specimen, the SFM image contains for every point of the surface all three spatial coordinates (x, y, z). This is the basis for a variety of image processing, yielding, for example, the height or the spatial arrangements of surface features. Another interesting data set would be the volume of investigated specimens. In structural biology, volume changes in relation to the environment (e.g., hydration state, ion concentration) or mechanical interactions (e.g., dissection) are valuable information, especially in the nanometer range.

Volumes of biological specimen can be characterized based on a reconstruction from a stack of serial cross sections [for a review see (Stevens, 1994)], e.g., collected by magnetic resonance imaging or confocal microscopy; or serial sections from the transmission electron microscope (TEM). Difficulties with this approach arise from the use of virtual or real sections, which should be ideally as thin as the highest resolution. Further problems are connected with the bleaching of fluorophores in confocal microscopy or the inhomogeneity and alignment of TEM sections.

Volume information derived from SFM images were used for the estimation of the water content of sperm nuclei (Allen, 1991). The stoichiometry of protein complexes bound to DNA was determined using relative volume comparison from SFM data (Wyman *et al.*, 1995). The volume of biomolecules based on the cross-sectional area was used to characterize elastic deformation of samples (Lyubchenko *et al.*, 1993). Such approaches were indirect. They were based on approximations for the shape of the molecule of interest and the use of dimensions estimated from SFM images.

Here we present a direct approach to calculate the apparent volume of specimen imaged by SFM, using the three-dimensional image information. Calculations from sets of human metaphase chromosomes are presented

and compared with results obtained by conventional methods. A possible application for classification of human chromosomes using the relative volume is shown. The accuracy of the algorithm as well as inherent problems (e.g., tip convolution, drying artifacts) are discussed.

Recently, an algorithm similar to those presented in this paper was used for the volume determination of plant metaphase chromosomes (McMaster *et al.*, 1996). They found a correlation between the volume and the classification of maize and barley chromosomes.

Materials and Methods

Metaphase chromosomes

Standard cytogenetic spreading methods were used for preparation of metaphase chromosomes from human lymphocytes, including hypotonic swelling and fixation in methanol-acetic acid (3:1) prior to spreading on a glass substrate (Harrison *et al.*, 1981). The spreads were usually imaged in the week they were prepared.

SFM imaging

SFM data were collected using a BioScope [Digital Instruments (DI), Santa Barbara, CA]. Contact mode images of metaphase chromosomes were obtained in the topographic (isoforce) mode. Scanning was performed using a J-Scanner (DI) with a 100 μm scan range using standard pyramidal shaped Si_3O_4 tips (DI). Image processing and analysis were carried out using the NanoScope software and NIH-Image 1.49 (NIH, Bethesda, MD). For volume determinations, images of the complete metaphase spread were used, with typical scan sizes of about 40-60 μm.

For rehydration experiments, metaphase chromosomes were imaged in air (35-50% relative humidity), and again after immersion in aqueous buffer (phosphate buffered saline, PBS), as described previously (Fritzsche *et al.*, 1994). Imaging started about 5 minutes after rehydration. The vertical force applied by the tip was minimized by stepwise lowering of the set-point to the point shortly before the tip loses contact with the surface. The dry volume of chromosomes was set at 100% for a graphical comparison with the rehydrated volume.

Volume determination

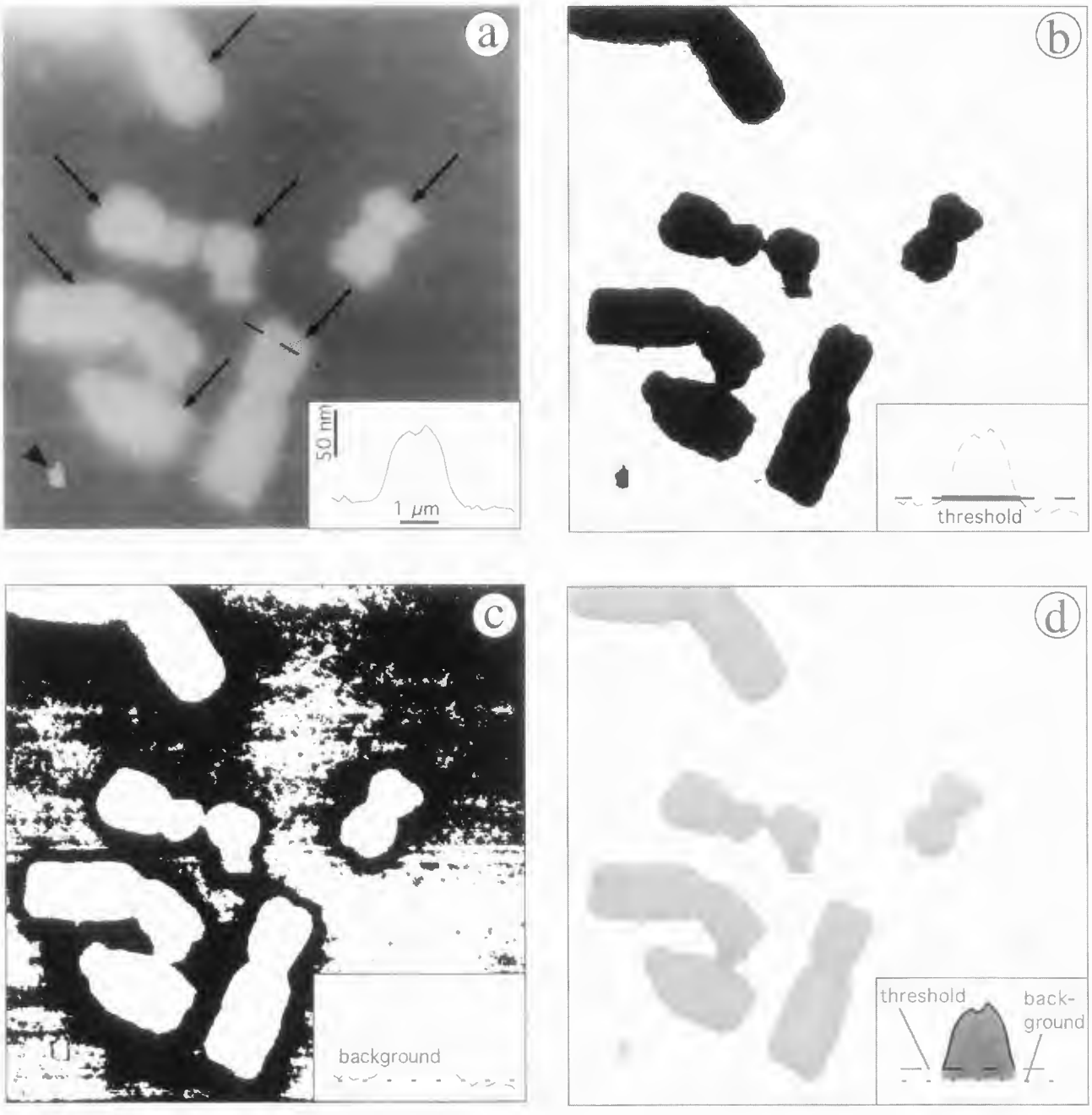
The apparent volume of a specimen adsorbed on a flat substrate was estimated from the SFM image by integrating all volume elements (voxel) below the specimen surface and above the substrate level, assuming close contact with the substrate surface. NanoScope images (top view) were exported in TIFF format and opened in NIH Image (NIH, Bethesda, MD). Height range and scan size are known from the SFM image, and were aligned with the range of pixel brightness and pixel number allowing the volume calculation of one volume element (voxel) with the dimension of one pixel in x, y, and z. The TIFF files cover a height range of 8 bits (256 steps). 239 steps are used for the height range of the SFM image, and the remaining values are used for other purposes. The threshold value for cutting off the specimen structure was chosen interactively using NIH Image. Starting with the highest feature (usually parts of the chromosome), the threshold level was lowered stepwise (i.e., bitwise) until background features started to appear beside the specimen structure. Then, the threshold was increased one step and saved as the final value. Subtracting all image points below the threshold created a mask (Fig. 1b), which was used for cutting off the specimen structure. The background level was determined as the average of all background height values (after removal of the specimen structure by masking, Fig. 1c), then all height values of the background were zeroed. Integration about all height values of the remaining image yielded the sum of voxels as the volume of the specimen (shaded area, inset, Fig. 1d).

Figure 1 (on page 105). Volume determination based on SFM images. (a) Scanning force micrograph of metaphase chromosome spread (arrows), brighter pixels correspond to regions of increased height. A preparation artifact, presumably caused by salt residue or cellular debris, is marked by an arrowhead. The inset shows a cross-section of one chromosome along the dotted line. (b) To discriminate between specimen and background, a threshold is chosen interactively. The value should be higher than background features (cf. dashed line in inset). (c) Using the mask generated in (b), the specimen was removed from the image and the background level is calculated as the average height of the remaining areas (dotted line in inset). (d) The mask from (b) was used to isolate the specimen. The background level calculated in (c) is used to exclude the background from volume calculation (cf. inset). The shaded area in the inset represents the calculated volume.

Results

The application of the algorithm for volume determination is highly influenced by basic steps, including the specimen cut-off and the background determination. These steps are considered in the following section.

The threshold value for cutting off the specimen structure was chosen interactively. Such a procedure is, of course, influenced by other structures on the image of interest, e.g., salt crystals or cellular debris in case of the chromosome preparations. These artifacts can be removed from the image before thresholding by using image processing tools. Another feature interfering with



threshold determination is background tilt, which should be corrected by previous flattening. To examine the influence of the chosen threshold on the determined volume, a typical image of a metaphase spread was the subject of volume determination based on different thresholds (Fig. 2a). Typical ranges of threshold uncertainty are 1-3 steps, equivalent to about 1-4 nm in height (for typical height ranges of 300-800 nm). Threshold variations in this range result in volume variations of less than 5%.

The background level can be determined more accurately (compared to the subjective threshold estimation) by calculating the average value for the whole background. The determined background is stable against variations in the cut-off value (threshold), this value can typically vary by 5 steps without changing the calculated background, as shown in Figure 2b. The stability of background determination is an important feature because the resulting volume would be strongly influenced by changes of the background value (Fig. 2c).

The influence of scan size of the volume determination was tested by imaging chromosomes at different scan sizes (20, 40, and 60 μm, respectively; data not shown). The chromosome volumes varied by more than

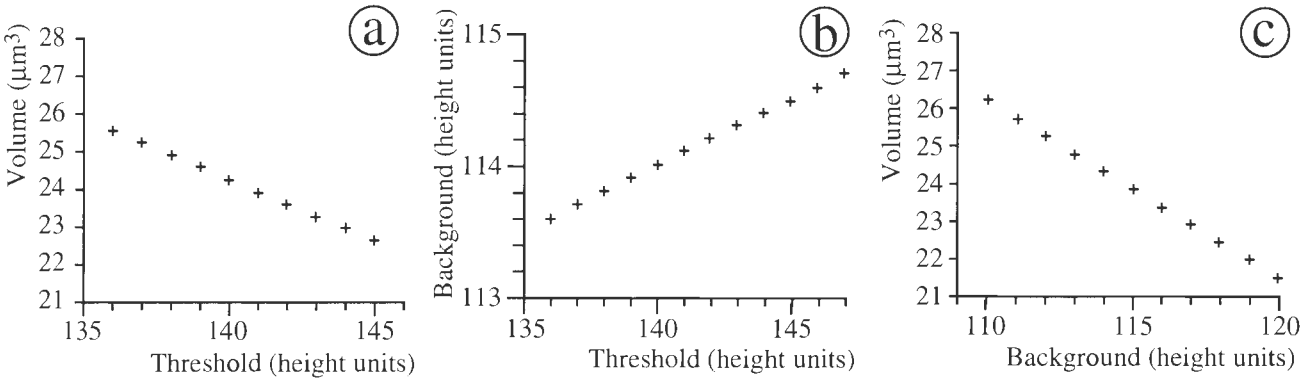


Figure 2. Influence of parameters on volume calculations, determined on a typical image (chromosome spread, scan size = 50 μm, height range = 400 nm). (**a**) Variation of the threshold level (cf. Fig. 1b) resulted in moderate changes of the volume. Typical ranges of uncertainty for the threshold level are 1-3 height units. (**b**) The determination of the background level is very stable, the normal range of uncertainty (1-3 height units) induces no significant change in the resulting level. (**c**) Changes in the background level would result in pronounced changes of the volume.

10%, but the ratio between the volumes was still preserved (variation of less then 6%). So, the scan dimensions seem to influence the measured absolute volume (presumably by losing resolution with bigger scan sizes), but the relative volume is unaffected.

Volume of metaphase chromosomes

Volume determination of complete metaphase chromosome spreads yielded 25.5 ± 3.4 μm^3 in the air-dried state (Fig. 3a). These spreads were selected, avoiding incomplete spreads (less than 46 chromosomes) and spreads with overlaying chromosomes. Spreads with the latter feature were occasionally found and resulted in a volume in the range of 18-25 μm^3. A sample with low surface stability (causing blurred images) yields volumes of 30-35 μm^3. After manual removal of major undesirable features in the images, the determined volume dropped down by 5-10%, still exceeding the volumes of spreads from stable scanned samples as mentioned above. A possible reason for the high volume (beside incomplete correction) could be amorphous salt covering the chromosomes, inducing the streaks in the image by instability against scanning by the tip.

The volume of single chromosomes varied between about 0.18 and 1.3 μm^3. In reference to the median set volume, these values are equivalent to 0.6-4.8% (e.g., Fig. 3b).

The behavior of chromosomes after rehydration was investigated. A swelling of the structure could be observed in SFM, as reported previously for chromatin fibers (Fritzsche *et al.*, 1995a) or metaphase chromosomes (De Grooth and Putman, 1992; Fritzsche *et al.*, 1994). The SFM-contrast was clearly increased due to the increase in height. In order to quantify the swelling effect, the volume in the dried state was chosen as reference value of 100%. After rehydration, the volume increased to 400-550%, determined from images under minimized vertical force (Fig. 3c).

Discussion

The scanning force microscope yields the topography of a surface, providing the information about the apparent volume of specimens. In contrast to conventional methods of volume determinations, which are based on multiple-step processes, the SFM data include the volume directly. This volume is influenced by the preparation and the measurement and should therefore be designated as the **apparent** volume of the specimen.

Simple air drying tends to result in flattening of the specimen, as in the case of metaphase chromosomes. So the SFM measurement of air-dried specimen should result in a decreased apparent volume compared to the native specimen. On the other hand, air-dried metaphase chromosome preparations are part of the standard classification procedures. The classification (karyotyping) of chromosomes for cytogenetic (including clinical) applications is based on morphological features and banding patterns, induced by cytochemical procedures and routinely visualized by optical microscopy [for a review see (Ford, 1973)]. Therefore, we believe that air-dried metaphase chromosomes are a suitable preparation for SFM-based classification studies. In particular, the use of the relative volume should minimize the effect of parameters which influence the appearance of a chromosome spread in SFM. The relative volume of chromosomes (ratio of volume to the volume of a whole set) combined with the centromere volume index (ratio of volume of the short arm to the chromosome's total volume) is specific for each chromosome, as studied using

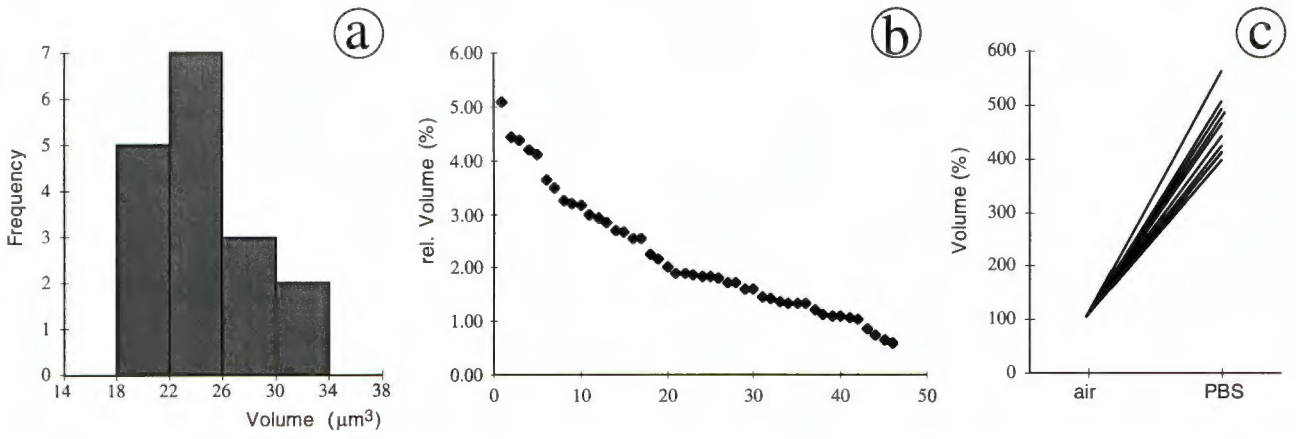


Figure 3. Volume of spread human chromosomes. **(a)** The volume of whole sets of human chromosomes results in 25.5 ± 3.3 μm^3. **(b)** The relative volume of every chromosome of a selected spread (ratio of the individual chromosome volume to the volume of a whole set), ordered according to the volume. **(c)** Hydration-state dependence of chromosome volume. The dry-volume of 9 randomly selected chromosomes of one spread was measured on air and referred as 100%. After rehydration in aqueous buffer, a volume increase of 400-550% was observed.

TEM serial sections (Heslop-Harrison *et al.*, 1989). Tip convolution effects, resulting in broadening of structures by SFM, should affect all chromosomes in a similar manner, and therefore, be corrected by use of the relative values.

Another effect influencing chromosome volume is the preparation procedure. Since all chromosomes have a similar composition, they should be influenced in the same way, preserving the relative volume of single chromosomes to some extent. The consistency of the DNA sequence is preserved, as the application of *in situ* hybridization to metaphase spreads demonstrate [e.g., (Baumgartner *et al.*, 1991; Rasch *et al.*, 1993)], and no difference between the structure of natively hydrated chemically isolated chromosomes (which have never been dried) and air-dried metaphase spreads was found in scanning electron microscopy (Allen *et al.*, 1985). This could be due to an inherent resistance of nucleic acids to damage by air drying (Sanchez-Sweatman *et al.*, 1993). Backed by these observations, we believe that the relative volume obtained from air-dried specimens is comparable to the relative volume data based on electron microscopy of embedded specimens.

The algorithm used for volume determination is explained in Figure 1. A typical problem is a rough or tilted background, as seen in Figure 1c. Small background differences (1-3 height units) have negligible influence on the determined volume. Preparation artifacts, such as salt or cellular debris (arrowhead in Fig. 1a), can be identified by structural parameters prior to removal by image processing. They have no influence on background calculation, because they are cut off by the masking procedure.

A critical point in the algorithm used here is the determination of the threshold level for cutting off the specimen from the background. A low value could include background features, whereas a high value would result in losing some parts of the specimen volume. In the case of the spreads used here, a value one height unit above the highest background feature (excluding artifacts like salt or debris) was chosen. Based on this value, the specimen was cut off and the background average was calculated. The resulting background level was then used to determine at which height unit the specimen starts (where the background ends). Because the threshold level was higher than the background average, some parts of the chromosome flanks are excluded from volume determination. By handling images of whole spreads, the loss will be similar to all chromosomes and not influence the relative volumes.

The apparent volume determination of metaphase chromosomes sets yields 25.5 ± 3.4 μm^3 (Fig. 3a), less than one fourth of the value determined by TEM studies of embedded chromosomes (Heslop-Harrison *et al.*, 1989). A main difference between both studies is the hydration state of the chromosomes. We investigated the chromosomes air-dried after spreading, whereas the TEM study used an embedding technique, which should preserve the native hydrated volume of the chromosomes to a large degree. Changing the hydration state of chromosomes by rehydration yields an 4-5 fold increase in volume (Fig. 3c), which is near the value found by the TEM study.

With relative chromosome volumes about 0.7-5.1%, we found a similar range as in the TEM by serial sectioning [0.86-4.41% (Heslop-Harrison *et al.*, 1989)].

The distribution of the relative volume (Fig. 3b) resembles the distribution of the relative volume based on serial sections (Heslop-Harrison *et al.*, 1989), relative length (ISCN, 1981), or DNA content by microdensitometry (Mayall *et al.*, 1984). These correlations support the applicability of the SFM technique used here for classification of human metaphase chromosomes.

Conclusions

We introduced a direct approach for volume determination of biological specimens. The application of the algorithm to metaphase chromosome spreads resulted in relative volume ranges and distribution comparable to values obtained by other techniques. Additional studies will be conducted to further test the application of the SFM method for chromosome classification.

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Discussion with Reviewers

P.W. Hawkes: The method you used to measure volumes is rather laborious and not convenient if a large number of such measurements are to be made. What improvements do you have in mind to speed up the process and increase the absolute accuracy?

Authors: The starting point for the volume determination should be one SFM image showing the whole set of chromosomes. In contrast to single images of every chromosome, we apply the same threshold and background criteria to every chromosome. This saves a lot of imaging time, which is still the most time-consuming part of the process. The resolution should be high enough to resolve the single chromosomes and determine

sufficiently exact values for the relative volumes. The next step is the separation of the single chromosomes prior to integration above the volume elements of the topographical image. At the moment, we do this separation by cutting out the area with a single chromosome and applying a NIH Image macro, which does the integration. We will improve the macro to handle the whole spread at once, so that one run will result in a list with the volume of each chromosome.

R. Lal: The height of the biological specimens, as measured by a scanning force microscope, is often smaller under aqueous condition compared to the dry condition. This is perhaps due to the deposition of buffers over the dry specimen surface and also because of the imaging force-induced compression of the hydrated specimens. If this is true for the chromosomes, the actual height of the rehydrated chromosomes may be overestimated. The authors should discuss this situation.
T.D. Allen: What does the actual image of rehydrated chromosomes look like in comparison to the dehydrated state?
Authors: We studied the height of several biological specimens, including metaphase chromosomes (Fritzsche *et al.*, 1994), chromatin fibers (Fritzsche *et al.*, 1995a), and microtubuli (Vater *et al.*, 1995), by comparing SFM images of the same biomolecule air-dried and then rehydrated. In every case, we observed an increase in height, presumably due to swelling effects. We do not fully understand the mentioned mechanism of buffer-induced compression of the specimen. In the case of the forces exerted by the scanning tip, we agree that there are elasticity effects, which are force-dependent and lower the height to some extent. We investigated such behavior on metaphase chromosomes, demonstrating that only strongly increased forces (about 3 times of the initial force) could squeeze the rehydrated chromosome down to the height of the air-dried state (Fritzsche *et al.*, 1994). Using minimized forces, rehydrated chromosomes are 4-5 times higher compared to the initial air-dried state (De Grooth *et al.*, 1992; Fritzsche *et al.*, 1994). However, we can not claim that the measured rehydrated heights are the true native heights.

R. Lal: The authors claim to measure the actual volume of the hydration state of the chromosomes by comparing the volumes of the air-dried and air-dried rehydrated chromosomes. The volume of the rehydrated chromosomes is reported to be similar to that obtained from earlier electron microscopic studies. The reasoning behind such comparison is not clear. The authors have reported an increased volume of the rehydrated chromosomes and argue that such an increase in the volume reflects the volume of the hydrated chromosomes which could occur when the chromosomes are embedded for TEM studies. For a normal EM embedding, biological specimen are invariably fixed and dehydrated. The authors need to provide some alternative reasoning.
Authors: For the TEM sectioning study, the cells were fixed, postfixed, then dehydrated, embedded, sectioned, stained, and finally EM imaged. This technique gives accurate data about chromosome size and morphology as *in vivo*, because it causes no deliberate physical or chemical distortion of the chromosomes (Heslop-Harrison and Bennett, 1984).

T.D. Allen: The basis of this paper is the value of SFM in determining accurate volume measurements of hydrated chromosomes. How do you know that re-hydrated chromosomes are the same as non-dehydrated chromosomes in the first place. Did you try chromosomes that have been (a) isolated and not dried, (b) cyto-spin without drying?
Authors: We used the volume of chromosomes from TEM serial sectioning studies [which should reflect the *in vivo* hydrated state (Heslop-Harrison and Bennett, 1984)] to get values for the non-dehydrated chromosomes. The agreement of these values with the measured volume of re-hydrated chromosomes demonstrated the similarity of both states in terms of the volume.

T.D. Allen: Is the resolution of the SFM significant to give us any indication of the fibrillar substructure at the surface of the chromosomes? The early SEM images suggest a chromatin fiber diameter of 0-50 nm, depending on how the chromosomes have been prepared.
Authors: The resolution of the SFM allows the visualization of biomolecules in the nanometer range, as demonstrated by various studies of DNA or other small biomolecules [for a review see (Hansma and Hoh, 1994)]. In case of the metaphase chromosomes, surface substructures with dimension of about 50 nm were reported on air-dried chromosome spreads (De Grooth *et al.*, 1992; Fritzsche *et al.*, 1994). Due to possible covering by cytoplasmic remnants and to damage, induced by the air-drying, these studies are not comparable with the SEM investigations using sophisticated sample preparations (Allen *et al.*, 1985). SFM studies using better preparation techniques and/or applied on partially decondensed chromosomes (Adolph *et al.*, 1977) should allow the three-dimensional visualization of the chromatin superstructure.

T.D. Allen: What was the diameter of the point of the tip used: does the edge interaction between the side of the tip with the edges of the chromosome as it approaches the substratum get taken into account? How do you account for the mobility of the probe to access the area

of the chromosomes which is between the central chromatid diameter and the coverslip: there is no way the tip can reach this space, presumably. Would a tapping mode scan be more accurate?

R. Balhorn: Did different types of tips interact differently with the chromosomes or did any type provide better images/data than another?

Authors: We are aware of the convolution between tip geometry and sample topography. The use of sharper tips should result in less exaggeration of the volume. Due to the flattened structure of air-dried chromosomes, the proportion of contacts made by the edge of the tip should be small, the structure of the swelled hydrated chromosomes are more affected. In the latter case, the volume is also force dependent, so reducing the imaging force by using tapping mode should result in a more reliable value for the volume. Experiments comparing the volume of rehydrated chromosomes using contact and tapping mode are on the way. However, we want to establish a classification procedure on relative volumes of the chromosomes, so that every effect which affects the volume of all chromosomes equally can be neglected.

R. Balhorn: This paper would benefit considerably if the authors could generate and include a plot showing the relationship between measured volume for each human chromosome and known values for their DNA content? This might allow them to determine if the extent of chromatin compaction for each chromosome is similar and demonstrate a potentially useful extension of the technique for investigating chromatin organization. Differences might be observed for chromosomes in which the centromeres make up larger portion of the chromosome or when comparing the Y chromosome or inactive X chromosome with the others, suggesting potential differences in chromatin compaction in inactive or functionally different chromatins that comprise metaphase chromosomes.

R. Lal: For this method to be applicable for a general chromosome classification purpose, there need to be additional studies comparing the volume of several different chromosomes.

Authors: This paper introduces the volume determination of metaphase chromosomes and establishes the application of the algorithm. The next step will be to classify the chromosomes after relative volume determination, in order to relate the volume information to the specific chromosome with a specific parameter (e.g., length, DNA content).

T.D. Allen: A typical spread of metaphase chromosomes lies in the cytoplasmatic remnants of the burst cell, did you take this into account with respect to your "base line" level of measurements by reference to areas of the substratum without spreads?

R. Lal: There appears to be some subjectivity in the determination of the threshold. The authors need to elaborate this point. Also, if it is not time consuming, this reviewer will recommend to calculate the volume of the background from the regions not covering the chromosomes: one can measure the background height directly by force-dissecting a small region of the substrate.

Author: The cytoplasmatic remnants in fact influence the volume determination. We do not know if this layer is below and/or above the chromosomes. If it is only below, we would be most accurate if we use the layer as background level (as we did in the paper). All other possibilities result in an exaggeration of the volume. Due to the small dimensions [average height is 7-9 nm (Fritzsche *et al.*, 1995b)] compared to the chromosome height, this volume increase should be small. However, as mentioned above, every influence that changes all chromosome volume equally is negligible due to the use of the relative volume. These points are also valid for threshold variations.

T.D. Allen: Is it possible to use this data to produce a contour of a single chromosome, e.g., diameter of centromere region relative to the p and q arms and their chromatids?

Authors: The result of the image processing procedure described in this paper creates a volume map of the entire chromosome. Therefore, the contour of the whole chromosome or of single chromatids is accessible, as is the height or width of the structure at different locations. However, we have not yet exploited the information in the context of chromosome classification.

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