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Use of surface affinity enrichment and cryo-embedding to prepare in vitro reconstituted mitotic chromosomes for EM tomography

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Abstract

We present a novel preparation method for studies of in vitro reconstituted mitotic chromosomes from *Xenopus laevis* egg extracts. This method involves a gentle adsorption of chromosomes from the extracts using surface affinity enrichment, followed by plunge freezing, freeze-substitution and cryo-embedding before examination by EM tomography. For comparison, chromosomes were also prepared by a conventional method, which included immobilization of chromosomes in agarose and a room-temperature dehydration/embedding protocol. Three-dimensional reconstructions showed that samples prepared with the new method have a greater interconnectivity of sub-structural features and a higher compaction ratio together with an apparently less perturbed chromatin structure than those prepared using the conventional approach. The implications of the new method for the preparation of other difficult samples and additional application possibilities are discussed.
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1. Introduction

One of the fundamental processes during eukaryotic cell division is the segregation of the duplicated genetic material in mitosis. This process involves the gradual condensation of chromosomes to single entities, the accurate positioning of the condensed metaphase chromosomes on the metaphase plate, and the separation of complete sets of chromosomes into daughter cells. Mitotic chromosomes have been the subject of structural research over the last 30 years, yet their

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higher-order organization remains uncertain. These studies have suggested the existence of a wide variety of characteristic substructures, e.g. fibers of a particular size [1–5] or larger aggregations [6]. While it has been proposed that fully condensed mitotic chromosomes arise from the hierarchical assembly of defined substructures [1,7-8], the details remain uncertain and no comprehensive model of higher-order chromosome organization has yet been developed. In fact, the most popular model, predicting the organization of 30 nm fibers into large loops that are anchored to a central chromosomal scaffold [9–14], is largely based on biochemical studies or observations of extracted or otherwise perturbed chromosomal material.

To better understand the structural changes occurring throughout the entire process of chromosome condensation, we have utilized the ability to reconstitute chromosome condensation in *Xenopus laevis* egg extracts. Extracts from *X. laevis* eggs are being used to study increasingly complex cellular processes in vitro ranging from chromosome replication and condensation to spindle formation and centrosome duplication. In the case of chromosomes, metaphase-arrested egg extracts can convert demembranated sperm nuclei to highly condensed single chromosomes having an overall appearance similar to metaphase chromosomes [15]. This led to the first identification and cloning of a protein complex (condensin) essential for chromosome condensation [16,17]. The advanced biochemical characterization of *X. laevis* egg extracts together with the accessibility of the reconstituted chromosomal material makes this system particularly attractive for chromosomal structural research. For example, essential components such as the condensin proteins may be immunolocalized in reconstituted chromosomes to determine their organization, or their function may be assayed by examining the structural consequences of depleting individual components from the extracts. Furthermore, because the condensation process is highly synchronized in the extracts, it becomes straightforward to examine the kinetics of the reaction. In addition, the condensation reaction can be also done with defined DNA templates of smaller sizes [18], facilitating the structural analysis of hierarchical assemblies of chromosomal substructures.

It is well appreciated that the method by which biological samples are prepared for electron microscopy can significantly influence the resulting structural observations. Chromatin and the ultrastructure of chromosomes are remarkably sensitive to perturbations in the ionic and chemical composition of buffers [19,20], making the preparation method a critical factor in the structural preservation of chromosomal material. Conventional preparation methods for EM studies usually involve a fixation step followed by dehydration of the sample and substitution with a polymerizing resin. Fixation, dehydration and embedding may all cause damage to chromosomal samples and various approaches have been developed in an attempt to minimize perturbation: critical point drying [1,7] to avoid embedding the sample, high-pressure freezing or plunge-freezing in combination with low-temperature dehydration/embedding to reduce the detrimental effects of dehydration and embedding at room temperature [3,21], or vitrification by rapid cooling and direct examination of the sample [5,22]. While vitrification and analysis by cryo-tomography is probably the most desirable strategy for optimizing structural preservation, it was not practical for our studies due to the thickness of the reconstituted chromosomes and the scarcity of the samples in the *X. laevis* egg extracts. To date, plunge freezing and cryo-tomography are best employed on samples, which are thin enough to be directly visualized and sufficiently abundant to be readily found [23,24]. The condensation process generally produces small clusters of entangled chromosomes that are larger than the 3–4-μm thickness compatible with a direct visualization in cryo-tomography. Although cryo-sectioning of the sample is possible, the method is best for 50 nm sections [25,26] and requires skillful expertise not readily available. In addition, the quite low number of chromosomes available in the preparations required an enrichment step together with a method for their easy identification within the sections, which would be very difficult in cry-sections. In order to resolve these problems, but to include the benefits of vitrification, we have opted for a preparation

method that results in embedded, sectioned material in which the chromosomes could be first identified in the section using fluorescence microscopy and then further examined in the EM.

We describe here a preparation method that was specifically developed for examining the ultrastructure of in vitro reconstituted mitotic chromosomes by EM tomography. This method has two novel components: the ability to gently concentrate chromosomes by surface affinity enrichment in a format compatible with plunge freezing, and a fast, low-temperature dehydration and embedding procedure to optimize sample preservation. The resultant semi-thick plastic sections were rapidly screened using fluorescence light microscopy to identify the location of chromosomes, and subsequently stained to provide high-contrast images for room-temperature tomographic data collection. We show three-dimensional reconstructions of samples prepared with this method and compare them with samples prepared in a more conventional way. Judging by the sharpness of the chromosomal borders, the connectivity of substructural features, and the uniformity in the packing, the new method appears to significantly improve the preservation of mitotic chromosome ultrastructure.

2. Materials and methods

2.1. Reconstitution of mitotic chromosomes with metaphase-arrested extracts from X. laevis

Metaphase-arrested *X. laevis* egg extracts and demembranated *X. laevis* sperm nuclei were prepared as described [27,28], flash-frozen and stored as aliquots at $-80\,^{\circ}$C. For the reconstitution reaction, the extract was diluted 1:1 (v/v) with energy-recycling buffer containing 10 mM HEPES pH 7.8, 50 mM sucrose, 0.1 mM $CaCl_2$, 5 mM EGTA pH7.8, 5 mM $MgCl_2$, 2 mM ATP, 20 mM phosphocreatine (Boehringer Mannheim, Indianapolis, IN) and 0.2 mg/ml creatine kinase (Boehringer Mannheim). Demembranated frog sperm nuclei were diluted with $1\times$XBE2 buffer (10 mM HEPES pH 7.8, 50 mM sucrose, 0.1 mM $CaCl_2$, 5 mM EGTA pH7.8, 2 mM $MgCl_2$) and added to the extract to a final concentration of 500 nuclei per µl extract. The reconstitution reaction was monitored by removing small samples and visualization with fluorescence microscopy.

2.2. Preparation of reconstituted mitotic chromosomes for electron microscopic studies

2.2.1. Conventional embedding method

In the initial stages of this study, significant emphasis was placed on optimizing sample preservation using conventional fixation, dehydration and embedding protocols, resulting in the following protocol. Low temperature agarose embedding was used to physically stabilize the chromosomes, greatly improving their ability to survive the methods we employed in sample handling. Chromosomes were strongly fixed in the reconstitution solution by addition of 1 vol 8% formaldehyde solution in $1\times$XBE2 buffer and incubation for 15 min, followed by addition of 0.5 vol 7.2% glutaraldehyde solution in $1\times$XBE2 buffer and incubation for 1 h. A 400 µl chromosome suspension was carefully layered on top of a two-step sucrose gradient (upper phase: 400 µl 30% isotonic sucrose, lower phase: 150 µl 66% isotonic sucrose cushion) in a siliconized 1.5 ml Eppendorf tube. After centrifugation ($5600\times g$/HB-4 rotor/15 min/ $4\,^{\circ}$C,) a hole was punched in the bottom of the tube and the chromosomes at the interface between the sucrose cushions were eluted dropwise and stored on ice as single fractions. Fractions containing the largest number of chromosomes as determined by fluorescence microscopy were combined (total volume ~15 µl) and quickly mixed with 4 µl 3.6% OsO_4 (Ted Pella, Redding, CA) in $1\times$XBE2, 1 µl 4% Cibacron Blue immobilized agarose bead solution (Pierce, Rockford, IL) as visual markers and 20 µl 4% low-melting-point agarose (Sigma, St. Louis, MO) in $1\times$XBE2 pre-warmed to $42\,^{\circ}$C. The mixed solution was placed on ice for 4 h to ensure complete polymerization of the agarose. Small agarose blocks were dehydrated on ice in an ethanol gradient (10% increments; incubation 30 min per step). After dehydration the agarose blocks were embedded in Epon (Poly Bed 812 Resin: dodecenylsuccinic anhydride: methyl nadic anhydride

13:8:7; Polysciences, Warrington, PA) by incubation in gradually increasing concentrations of Epon solutions (Epon:ethanol 1:3, 1:2, 1:1, 2:1, 3:1 with incubation 1 h per step) to 100% Epon and finally incubated for 24 h at 45 °C, and for 48 h at 65 °C for complete curing of the resin.

2.2.2. Surface affinity enrichment/cryo fixation method

2.2.2.1. Surface affinity matrix preparation. A polymer of methylmethacrylate and methacrylic acid in the ratio of 9:1 (Polysciences) was dissolved in dichloromethane (Sigma) to 5% concentration with 0.375% dibutylphthalate (Sigma) as plasticizer to reduce the stiffness and brittleness of the plastic. Approximately 20 ml of the solution were poured in a glass petridish (diameter 6 cm) and thin, visually transparent plates of solid matrix were obtained by slow evaporation of the organic solvent. Small handles (half a tabbed EM grid) were glued to matrix pieces of adequate size to facilitate further handling. The plastic matrix surface was activated with 0.2 M 1-ethyl-3-(3-dimethlaminoprophyl) carbodiimide (EDAC; Molecular Probes, Eugene, OR)/ 0.05 M *N*-hydroxysuccinimide (Sigma) to be linked with a protein construct consisting of three identical domains of the DNA binding motif (amino acids 378–431) of the human telomere binding protein TRF1 [29] connected with a linker of 15 glycine residues. The his-tagged protein was expressed in *Escherichia coli* using vector pET-16b (Novagen, Madison, WI) and purified under denaturing conditions as recommended with the exception that it was renatured while bound to the nickel-nitrilotriacetic acid chromatography column (Qiagen, Hilden, Germany) with a 6–0 M guanidine gradient in buffer containing 25 mM sodium phosphate pH 8.0, 0.5 M NaCl, 0.1 mM benzamidine. After imidazole elution and dialysis against 20 mM Tris pH 8.0, 0.2 M NaCl, 5% glycerol, 0.5 mM benzamidine, the protein was purified further on a SP-Hi-Trap-column (Pharmacia, Uppsala, Sweden) using a 0.1–1 M NaCl gradient in 50 mM Tris pH 8.0. The presence of the construct was confirmed by sequencing of the expression vector, Western Blotting with anti-his-tag antibodies, and by checking its DNA binding activity with oligonucleotides containing the telomeric binding motif. For coupling to the activated surface of the plastic the protein was diluted to 50 μM with 0.025 M sodium phosphate pH 8.25, and incubated on the surface for 1 h. Non-reacted carboxyl groups on the matrix were neutralized by incubation in 0.025 M sodium phosphate pH 8.25, 0.5 M glycine for 30 min. To reduce non-specific association to the surface the plastic was further incubated with buffer containing 1 mg/ml BSA and 50 μg/ml tRNA (Sigma). The binding capability of the protein on the surface was again confirmed with oligonucleotides containing telomeric sequences (estimated binding capacity 1000 protein molecules/μm^2).

2.2.2.2. Cryo-fixation, freeze-substitution and cryo embedding. Reconstituted chromosomes were initially fixed by addition of 9 vol 0.5% glutaraldehyde/1% formaldehyde in 1 × XBE2 to the extract and incubation for 15 min at room temperature. Prepared plastic matrix pieces were placed on a silicon plug at the bottom of a centrifuge tube (SS-34, Sorvall) and 2 ml 33% sucrose in 1 × XBE2 added on top of the matrices. The fixed chromosome suspension was carefully layered on the sucrose cushion and centrifuged (5600 × g, HB-4 rotor, 10 min, 4 °C). The plastic pieces were recovered and incubated for 10 min in the same fixative as above at 4 °C. After removal of excess liquid from the matrix with filter paper the plastic pieces were immediately plunge-frozen (plunge freezing device from precision engineering EMBL) in liquid ethane. For dehydration, the plastic pieces were transferred under liquid nitrogen to 1.5 ml Eppendorf tubes containing 1 ml pre-cooled methanol and incubated at −80 °C for 2 h. The plastic pieces were washed twice with pre-cooled acetone at –80 °C and then incubated for 1–2 h at −80 °C in an acetone solution containing 7.5% poly(methylmethacrylate) (Polysciences) and 0.375% dibutylphthalate for substitution. Subsequently the pieces were quickly placed on a microscope slide on a metal block pre-cooled with dry ice to −80 °C immersed in an excess of substitution solution. The remaining organic solvent was removed by applying a vacuum over 12 h in a desiccator cooled to −80 °C, thereby

producing an additional plastic layer on top of the samples. To ensure the stability of the plastic matrix all organic solutions, tubes and pipetting devices must be kept at dry ice temperature during this process. For the same reason, the concentration of the methacrylic resin in the substitution solution should correspond to that of a fully saturated solution at $-80\,^{\circ}C$, which may vary depending on the grade of the acetone solvent. The encapsulated samples were warmed to room temperature and re-embedded in Epon blocks.

2.3. Staining of sections

Sections of 0.2–0.4 μm in thickness were placed on formvar copper or finder grids (Ted Pella). Despite the addition of dibutylphthalate as plasticizer, the acrylic resin blocks are substantially harder than Epon blocks, requiring a frequent exchange of glass knives. The samples were first stained with an aqueous 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma) solution for identification of the position of chromosomes in the section using fluorescence microscopy. Grids were subsequently positively stained with 0.25% uranyl acetate in 50% ethanol for 45 min and 0.02% lead citrate in 10 mM NaOH for 10 min for analysis in the EM. Samples were choosen for data collection if their overall structure was representative for all identified chromosomes, if they were well stained, and if their location on the grid allowed high-tilt series tomography. For data collection one surface of the grid was treated with poly-L-lysine followed by attachment of 20 or 30 nm gold beads (Ted Pella) as fiducial markers and carbon coating to minimize charging in the electron beam.

2.4. EM data collection, data processing and data analysis

EM tomography allows a three-dimensional image of the sample to be reconstructed from a tilt series of medium thick sections [30]. Tomographic tilt series were collected on a Phillips 430 electron microscope equipped with a CCD camera using software as previously described [31] at 300 kV with 17,100 × or 21,200 × magnification. Single-tilt series were automatically recorded using a high-tilt goniometer holder (Gatan) with a tilt angle increment of 1.25° and a tilt angle range typically of $\pm 60^{\circ}$ to $\pm 70^{\circ}$ One data set was recorded on a Phillips T20 microscope at 200 kV using a tilt angle increment of 1° with 14,500 × magnification. As is common practice in EM tomography of plastic sections prior to data collection, the samples were exposed to the electron beam for 0.5 h enabling the vast majority of the beam-induced section shrinkage and sample damage to occur before, rather than during, data collection [32]. Gold bead alignment and reconstruction was done using the Priism software [33]. The data sets were recorded with nominal resolutions of 2.05 nm/pixel and 1.68 nm/pixel on the Phillips 430 microscope, and 1.5 nm/pixel for the data set collected on the T20 instrument.

The average radial power spectra were calculated by Fourier transformation of individual z sections and subsequently averaged over all z sections in the sample volume. Tendency curves were fitted by weighted smoothing using Kaleidagraph (Adelbeck software; 1993).

2.5. Fluorescence light microscopy

Aliquots of the reconstitution reaction were mixed 1:1 (v/v) with buffer containing 16% formaldehyde and 10 μg/ml DAPI. The samples were visualized with a conventional fluorescence microscope (Zeiss III RS). For three-dimensional light microscopy, the chromosomes were reconstituted and fixed by addition of 1 vol 1% glutaraldehyde/ 2% formaldehyde in 1 × XBE2 for 5 min followed by addition of 1 vol 2% ultra-low melting agarose (Sigma; pre-warmed to 37 °C) in 1 × XBE2 containing 0.25 μM YO-PRO (Molecular Probes). Staining with YO-PRO (emission 491 nm, excitation 509 nm) consistently gave images with less blurring in comparison to DAPI (emission 358 nm, excitation 461 nm). A small aliquot was placed on a slide, and the agarose was allowed to polymerize for 10 min at on ice after a cover slip was applied. Three-dimensional data sets were recorded with a confocal microscope (Leica TCS NT).

3. Results

3.1. Reconstitution of mitotic chromosomes in vitro

A time series of the reconstitution of *X. laevis* sperm nuclei to mitotic chromosomes using a metaphase-arrested *X. laevis* extract is shown in Fig. 1.The highly compact sperm nucleus containing a haploid set of 18 chromosomes (Fig. 1a) undergoes a rapid swelling phase (Fig. 1b), followed by continuous remodeling and appearance of very fine structural details (Fig. 1c) and the continuous gradual compaction and separation of chromatin until individual condensed chromosomes either completely separated or entangled in small clusters are recognizable (Fig. 1d). These

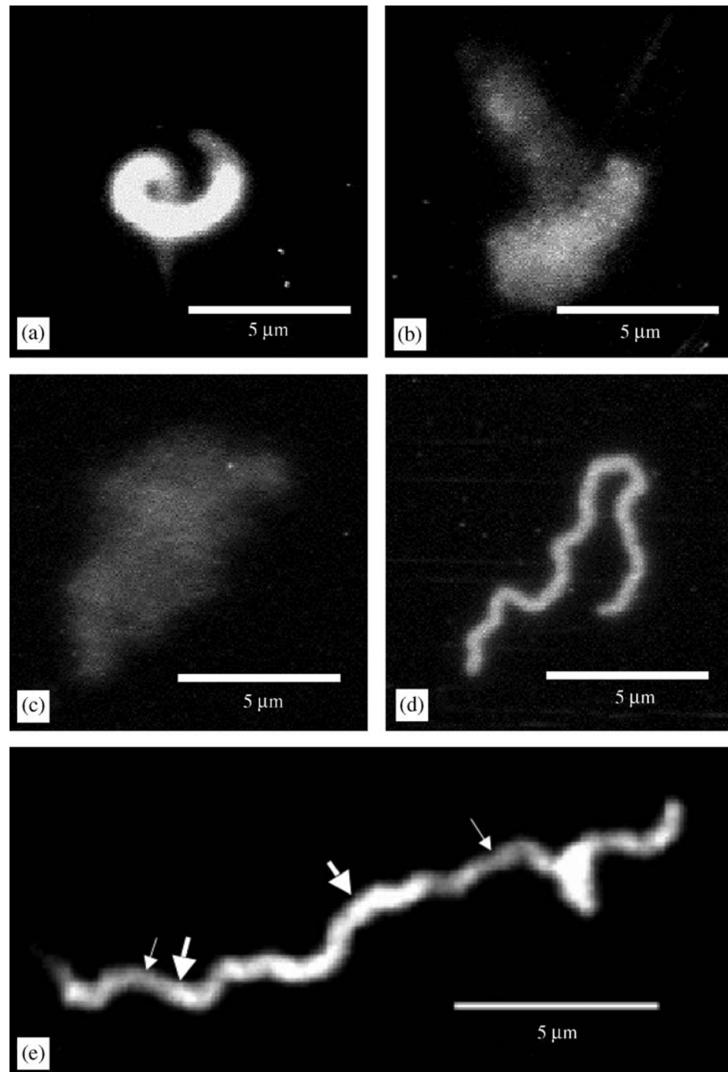

Fig. 1. Time series of the in vitro reconstitution reaction from *X. laevis* sperm nuclei to mitotic chromosomes. (a)–(d) samples were stained with DAPI and visualized with a conventional fluorescence microscope. The time points are (a) 0 min, (b) 10 min, (c) 30 min and (d) 120 min.(e) Chromosomes were incubated for 120 min and stained with YO-PRO. The three-dimensional data set was recorded with a confocal laser scanning microscope.

chromosomes (formally chromatids) are thin and elongated, with a typical length of between 20 and 30 μm and a diameter of approximately 0.3–0.5 μm. With the given resolution of the fluorescence microscope of approximately 0.2 μm, the reconstituted chromosomes have a smooth surface and show no indication of periodicity along the chromosome axis (Fig. 1e). However, variations in the chromosome diameter are apparent when looking at different segments of the reconstituted chromosomes (small and large arrows in Fig. 1e). This might indicate either an asymmetry in the cylindrical shape or a variable diameter of the chromatid arm. In general, the observed dimensions are characteristic of amphibian chromosomes at the early embryonal life stage [34], suggesting that the in vitro reconstitution reaction yields chromosomes with an appropriate overall morphology.

3.2. Sample preparation strategy

A fundamental challenge in preparing in vitro reconstituted chromosomes for EM tomography is that they are exposed to the surrounding medium without the protecting boundaries of the nucleus or the cell. In order to process them through the number of steps required until the final embedding in plastic, it is necessary for the chromosomes to be stabilized. This was previously accomplished by encapsulating chromosomes in small blocks of agarose [35] to facilitate handling. Here we use the agarose method in combination with chemical fixation as a ‘‘conventional’’ reference for comparison with our new method, below. After isolation of the chromosomes and embedding the chromosomes in agarose, the samples were further processed using conventional ethanol dehydration and substitution with Epon resin (Fig. 2a).

In the new technique (Fig. 2b), here referred to as the surface affinity enrichment method, lightly fixed chromosomes were gently sedimented through a dense isotonic sucrose layer onto a plastic matrix covalently labeled with a DNA binding protein. This protein consists of three tandem repeats of the DNA binding domain from the human telomeric binding protein TRF1 which binds to the conserved telomeric sequences at the end of vertebrate chromosomes. This affinity method was chosen over the use of poly-L-lysine in an effort to provide sample enrichment as well as to minimize potentially disruptive interactions with the matrix by localizing them just to telomeric regions at the ends of the chromosomes. We cannot rule out that once the chromosome is brought near to the surface, that non-telomeric DNA regions are also bound non-specifically by the protein construct. However, it seems reasonable to assume that a small DNA binding protein disturbs the ultrastructure less than a large polymer of positively charged residues. After binding of the chromosomes to the matrix, the sample is plunge frozen in liquid ethane. Since the chromosomes are relatively thin and exposed to the surface we expect a cooling rate that suffices for vitrification of the sample [36], resulting in optimal preservation.

Since the clusters of chromosomes are larger than the thickness that can be examined in the microscope, it was desirable to embed the frozen material for sectioning. Once plunge frozen, the sample was freeze substituted with methanol at –80°C. Since the chromosomes are still exposed on the surface they are quite delicate. To stabilize them, they were cryo-embedded in a thin coat of methacrylate by incubating with a poly(methylmethacrylate)-saturated acetone solution at –80°C. While maintaining the low temperature, the organic solvent was evaporated under vacuum, thereby encapsulating the samples in a thin layer of acrylic resin. The use of poly(methylmethacrylate) for room-temperature embedding has been previously reported [37]. In the low-temperature embedding protocol we took advantage of the high solubility of poly(methylmethacrylate) in acetone at −80 °C. The advantages of this embedding method in comparison to the commonly used Lowicryl low-temperature embedding [3] is that it is considerably faster, the temperature is kept at −80 °C throughout all stages of the processing (including embedding), and no chemical cross-linkers or long exposure to UV-light as in the case of Lowicryl are necessary. After embedding, the chromosomes can be visualized in the transparent plastic using a fluorescence microscope by addition

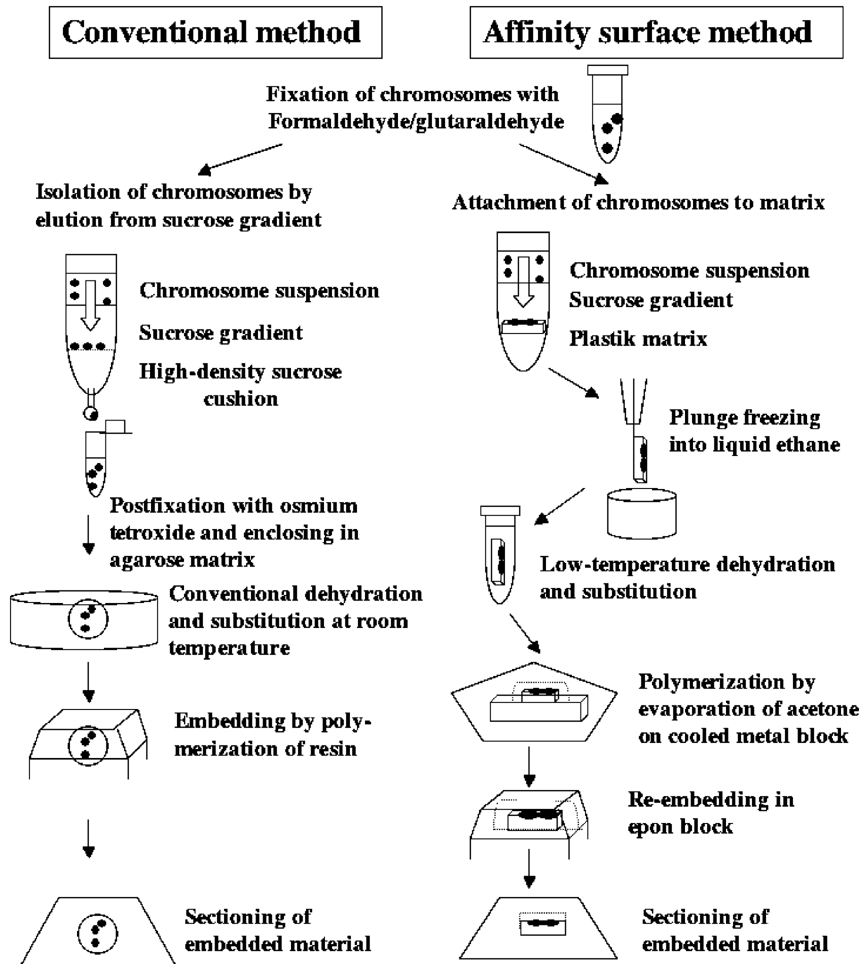

Fig. 2. Principal steps of the methods used to prepare in vitro reconstituted mitotic chromosomes for EM tomography. (a) Conventional embedding method (b) Surface affinity enrichment method.

of an appropriate dye during the embedding procedure (Fig. 3a; arrow). This greatly facilitates comparative analysis by light and electron microscopy methods. Here, we used the DNA-specific dye DAPI to visualize the location of chromosomes, thereby identifying suitable regions for EM analysis. Selected regions we cut out and re-embedded in a conventional Epon resin to facilitate sectioning. As shown in Fig. 3b, omitting the telomere binding protein results in a significant decrease in the number of chromosomes on a section, confirming the importance of the affinity adsorption approach.

3.3. *Comparison of the internal structure in three-dimensional reconstructions from EM tomography*

Semi-thick sections (0.2–0.4 μm) of samples from both methods were stained with uranyl acetate/lead citrate and tomographic tilt series were collected as described (Section 2). In Fig. 4a–d, the three-dimensional reconstructions of two typical samples from each method are shown for comparison. The samples display a related pattern of small fibrillar forms connected to a complex network. However, samples from the two preparation methods clearly differ in the

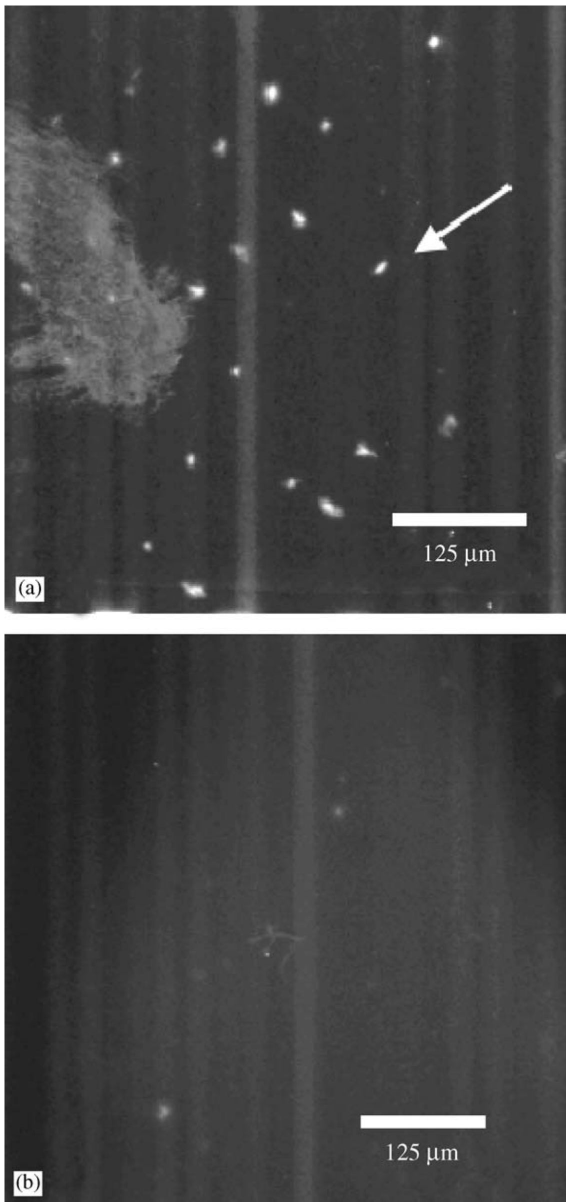

Fig. 3. Comparison of adsorption efficiency of chromosomes to plastic matrix after low-temperature embedding with poly(-methylmethacrylate). (a) Plastic matrix coupled with telomere binding construct. (b) Plastic matrix not coupled with telomere binding construct as control. In both cases, the shown pictures with an area of 0.5 mm × 0.6 mm correspond to representative areas of the middle of the approximately 2.5 mm wide plastic matrices. The chromosomes are visible against the background as small and brightly illuminating spots. Note the significant difference in chromosome adsorption.

degree of interconnectivity of the fibrillar forms as well as in the overall compaction density.

In general, samples prepared with the surface affinity enrichment method (Fig. 4a and b) appear to be packed more densely then those prepared by the optimized conventional method (Fig. 4c and d). This is indicated in a qualitative comparison of power spectra produced from samples from Fig. 4b and d, respectively (Fig. 5). In the sample prepared with the surface affinity enrichment method the characteristic point of the decline of the mean amplitude is shifted towards higher resolution (or smaller distances) (Fig. 5—label 'A'). This point corresponds approximately to the 'average' distance between larger sub-structural elements, hence to the overall density. From visual inspection we estimate an approximately two-fold higher compaction in chromosomes prepared by the surface affinity enrichment method, and this compaction typically extends throughout the chromosomes resulting in clearly defined outer boundaries (Fig. 4a and b). In contrast, chromosomes prepared by the conventional embedding method showed not only reduced compaction in the center of the chromosomes, but the packing density further decreases towards the outer periphery (Fig. 4c). The higher compaction seems to be directly linked to the degree of interconnectivity of the fibrillar forms indicating that essential but marginally stable interactions between sub-structural elements are preserved more effectively in the surface affinity enrichment method. In our view, it is likely that the observed interconnectivity is a consequence of a fundamental chromatin packing activity, therefore directly linked to chromosome condensation. The new preparation method apparently stabilizes this interconnectivity and should allow it to be studied in detail.

Another important criterion for judging the quality of the ultrastructural preservation is the integrity of single sub-structural features. The nominal resolution limit of the data sets presented here ranges between 2 and 3 nm in the x–y plane and approximately 4–6 nm along the z-axis allowing a detailed analysis of typical chromatin-like features, which are expected to be between 10 and 40 nm in size. This comparative analysis is significantly complicated by the observation that

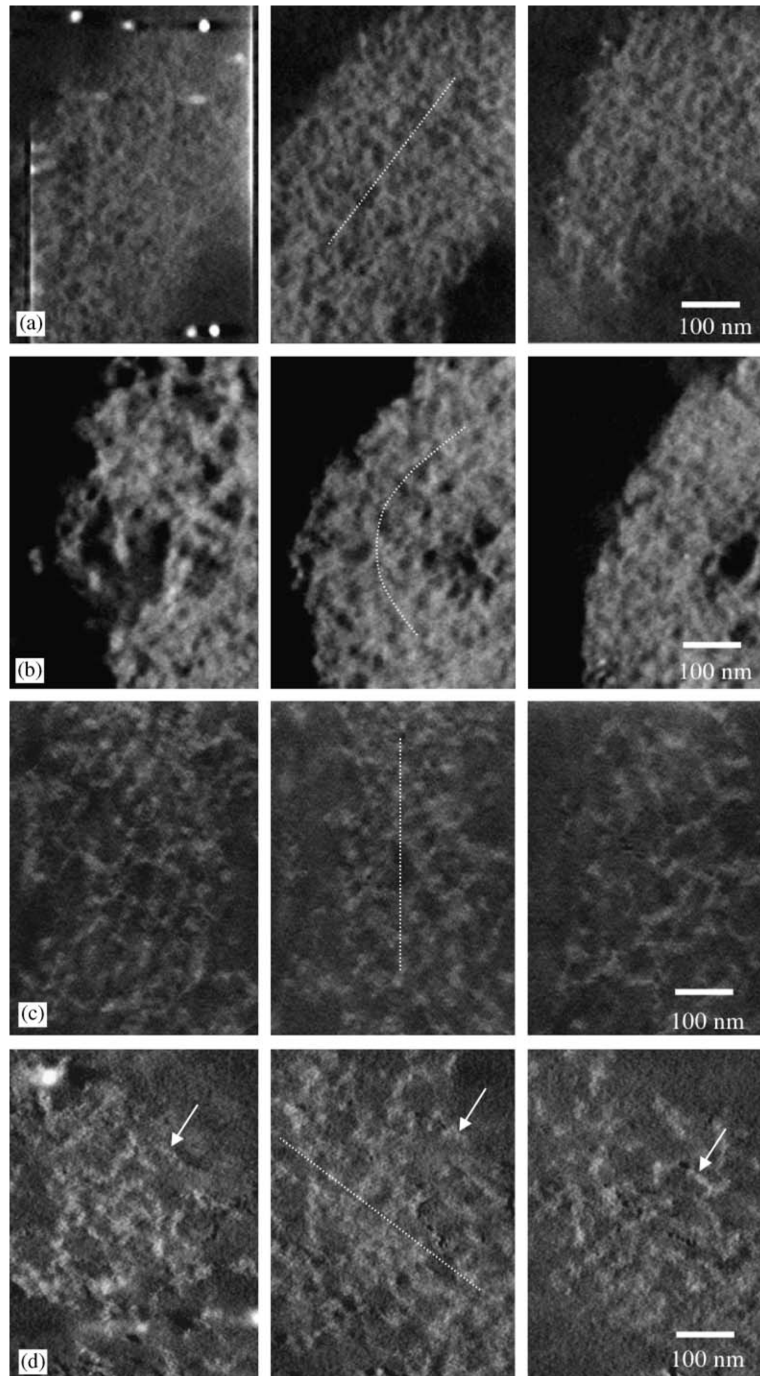

Fig. 4. Comparison of the internal structure of chromosomes in three-dimensional reconstructions from EM tomography. (a) and (b): Two different samples prepared with the surface affinity adsorption method. (c) and (d): Two samples prepared with the conventional method. The chromosomes in all samples are orientated approximately in the sectioning plane. For each sample, three sections are shown from regions throughout the chromosome. The central picture corresponds to the middle region of the chromosomes. The approximate path of the chromosomes in indicated by a dotted line. Note the significant difference in compaction.

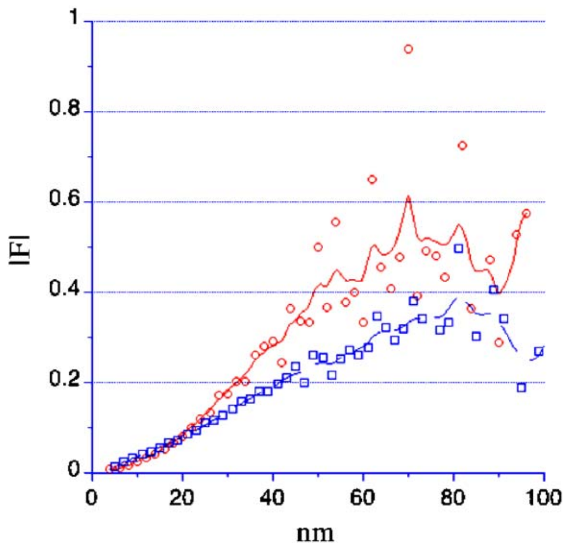

Fig. 5. Power spectrum of sample prepared with surface affinity enrichment method (circles) or conventional embedding method (squares). The absolute amplitude is plotted as function of spacing. Regions of interested are indicated and demonstrate the reduced compact in the conventional sample and the increase in unfolded chromatin-size structures.

we see in all samples a rather complex pattern of substructures, both in terms of size and shape, underlined by the absence of a characteristic dominant peak in the range between 10 and 40 nm in both power spectra (Fig. 5). The higher density of packing observed with the surface affinity enrichment method generally makes it more difficult to estimate the actual size of substructures. However, when comparing quantitatively the appearance of smaller substructures (e.g. in the range of 10–20 nm) we find that such small structures occur more frequently in samples prepared with the conventional embedding method (see small arrows in Fig. 4d). The slightly less pronounced decline of the power spectrum of samples prepared with the agarose matrix method may correspond to this observation (Fig. 5—label 'B'). Considering that the samples prepared with both methods were stained for EM in a similar manner and that the data collection methods were identical, this may suggest that the observed fibrillar structures in samples of the conventional embedding method of the size of around 10 to 15 nm correspond to more unraveled chromatin, probably related to beads-on-string models [38] and that this chromatin produces a weaker staining pattern than more compact structures seen in the surface affinity enrichment method. Therefore, the conventional embedding method has not only an effect on compaction and interconnectivity but also on chromatin structure.

3.4. The effect of the surface affinity enrichment method on the overall morphology of chromosomes

It is important to analyze whether the adsorption of chromosomes to a flat matrix in the surface affinity enrichment method may induce changes in the chromosome structure due to mechanical stress. While it is difficult to determine the effect on the internal chromosome structure, it may be possible to visualize stress-induced distortions of the overall chromosome shape. A typical chromosome sample in direct contact with the matrix surface is shown in Fig. 6a. The chromosome is almost completely contained within the boundaries of the section and the cross-sectional area of the chromosome is shown with a view exactly along the presumptive chromosome axis. The overall cross-sectional shape resembles that of an ellipsoid. This could either be the true shape of the chromosome, or could be the consequence of flattening of a cylindrical chromosome from either adsorption to the plastic surface or from beam-induced shrinkage. Epon sections show about 30% shrinkage [32], and preliminary measurements on the acrylic resin used here indicated that this resin is no less stable than Epon, and may well be more stable. The clearest way to separate preparation-induced distortions from intrinsic chromosome shape would be to find a chromosome arm that was perpendicular to the plane of the section.

Although the reconstituted chromosomes generally adsorb to the surface as single entities or loose clusters that flatten out, occasionally we found entire clusters of tightly interlinked chromosomes adsorbed to the surface. In these clusters, some chromosomes were not in contact with the surface and examples could be found where the chromosome arm was oriented almost perpendi-

cular to the matrix surface (Fig. 6b). Interestingly, the overall shape of the chromosome appeared to be still ellipsoidal, although slightly less severe. Similar asymmetrical shapes were seen in chromosomal samples prepared with the conventional embedding method (Fig. 6c) that are neither in contact with a matrix surface nor oriented in the direction of the expected section shrinkage. In conclusion, it appears that the reconstituted chromosomes are quite asymmetric in their natural state, and that this asymmetry orients the chromosomes as they adsorb onto the affinity surface (Fig. 6a). While additional flattening might occur, it is clear from these studies that this effect is small, making it difficult to quantify whether the natural ellipticity has been slightly exaggerated by sedimentation, adsorption or radiation damage.

4. Discussion

Our understanding of chromosome architecture depends greatly on our ability to preserve its structural integrity during sample preparation for electron microscopy. We compared the effects of two different preparation methods on the ultrastructure of in vitro reconstituted mitotic chromosomes from *X. laevis* in studies using EM tomography. In an optimized conventional embedding method, fixed chromosomes were isolated with a sucrose gradient followed by immobilization in agarose and dehydration/embedding at room temperature. In the surface affinity enrichment method, we combined gentle affinity adsorption of the chromosomes on a plastic surface with plunge freezing and low-temperature dehydration/embedding. We have observed that the reconstituted chromosomes prepared with the new method are better preserved based on criteria typically associated with the structural integrity of chromatin: namely, the interconnectivity between substructural elements, the degree of chromatin

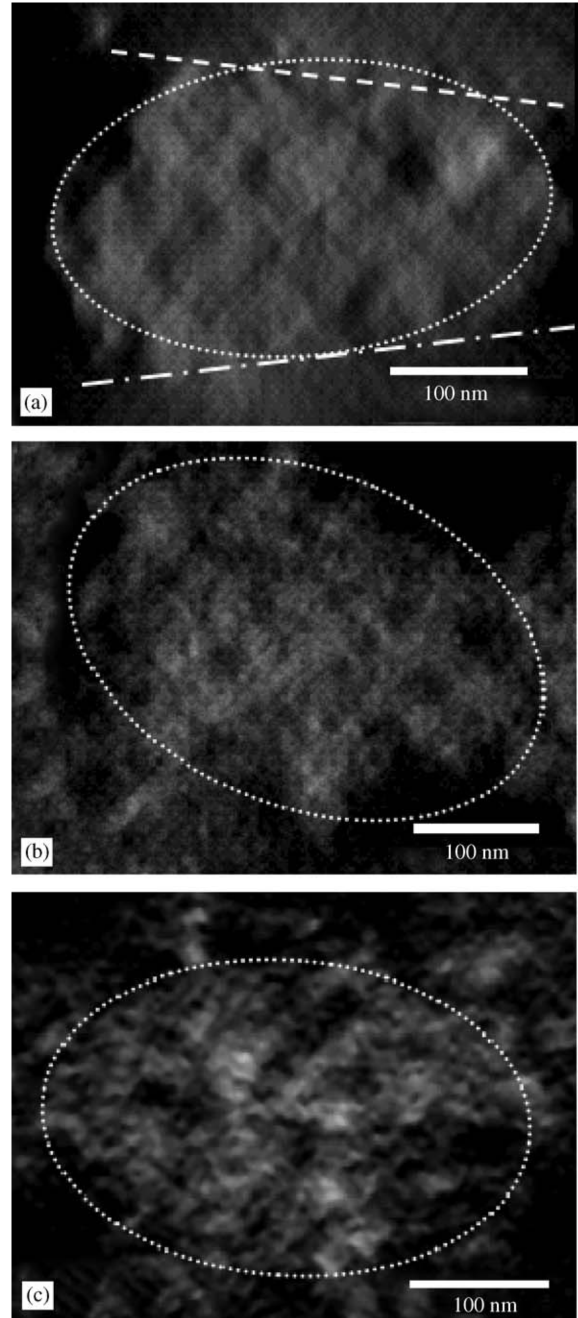

Fig. 6. Three-dimensional reconstructions of chromosomes viewed along the chromosomal axis. (a) Chromosome prepared with the surface affinity enrichment method in direct contact with the matrix surface. (b) Chromosome prepared with the surface affinity enrichment method not in contact with matrix surface. (c) Chromosome prepared with the conventional embedding method. The scale of magnification is shown with a bar. The outer shape of the chromosomes is indicated by dotted lines. In (a) the sectioning plane (- - -) and the plastic matrix surface (-.-) is indicated by lines.

compaction, the sharpness of chromosome borders and the reduced occurrence of unraveled and disintegrated chromatin [19].

The higher degree of interconnectivity of substructural elements observed in the samples prepared by the surface affinity method is most likely correlated with the significantly higher degree of chromatin packing. In general, chromatin packing is a very sensitive indicator for the structural integrity of mitotic chromosomes. Mechanical or chemical perturbations typically result in the loss of large-scale chromatin organization together with the appearance of more diffuse chromatin [19]. While it is possible that chromatin packing could be influenced by sample shrinkage during sample dehydration, it has been previously suggested that dehydration effects on chromatin are dramatically reduced in cryo-substitution methods [39]. Therefore, the observation that freeze-substituted chromatin is more densely packed than chromatin dehydrated at room temperature clearly argues in favor of improved sample preservation. Considering that both sets of samples were chemically fixed using formaldehyde and glutaraldehyde we conclude that the differences in observed interconnectivity and chromatin packing are likely due to the more effective preservation of marginally stable interactions between sub-structural elements by the plunge freezing and cryo-embedding in the surface affinity enrichment method. Although there is always the risk of incomplete vitrification and damage of the structure by local ice formation, this is unlikely to be a problem with such thin samples. Indeed, the remarkably more homogeneous and more compact structural appearance of chromosomes prepared by the surface affinity enrichment method argues that there is little or no significant ice damage.

The affinity adsorption of objects to a surface provides a relatively gentle way of enriching low concentration samples from complex extracts and it greatly facilitates localization of the chromosomes by light microscopy. It also facilitates cryo-preservation by plunge freezing. However, as with all adsorption approaches, it implies the risk of structural distortions occurring at the surface, especially if the sample is not sufficiently stabilized, for example by chemical fixation. Understanding the severity will depend on the sample and may involve either the comparison of objects bound to the surface in different orientations, or the comparison of structures obtained with complementary methods such as light microscopy.

5. Conclusion

While direct visualization of frozen hydrated samples undoubtedly represents the ultimate in specimen preservation, the low inherent contrast and low doses required makes data collection and sample handling more difficult. For thick objects that need sectioning, previously published efforts to improve preservation relied on plunge-freezing together with cryo-sectioning or high-pressure freezing in combination with low-temperature embedding. In general, these approaches are difficult to apply to isolated objects of the size of organelles present in only small numbers due to the challenges of sample handling, especially during low-temperature embedding. In the present method, the enrichment and immobilization of chromosomes onto a stable matrix together with a fast low-temperature embedding protocol avoids numerous practical problems associated with the processing of samples of this size for EM tomography. The method should be applicable to the studies of chromatin or chromosomes of other species and sources by EM tomography provided the samples can be isolated from the cell nucleus. In principle, any isolated object may be prepared with the described technique as long as it can be bound to the matrix, and if its size allows a sufficient cooling rate for sample vitrification. Attachment can be accomplished using specific binding proteins as described here, more generic reagents such as antibodies, or non-specifically by charged polymers such as poly-L-lysine. In addition to its use in the plunge-freezing procedure, the immobilization of the sample onto a matrix also offers the possibility of applying biochemical methods in a greatly simplified manner. For example, in case of the in vitro reconstituted chromosomes we applied a complete immunostaining protocol to matrix-immobilized

chromosomes to identify specific proteins by gold-conjugated antibodies in the three-dimensional reconstructions (König et al., manuscript in preparation). While the thick matrix used here provides convenient handling, if sectioning is not required, it should be also possible to adsorb the specific binding proteins or antibodies directly on the EM grid, followed by plunge freezing and examination under cryo-imaging conditions. Given the advantages of affinity enrichment, the techniques described here should provide a valuable aid to structural studies using EM tomography.

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