A novel approach to evaluate ultrathin section distortion

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INTRODUCTION

Compression of ultrathin sections of biological samples embedded in polymers strongly depends on the embedding protocol and the mechanical properties of the composite[1]. Regular diamond knives often deform ultrathin sections of hard biological specimens (i.e. pollen grains) embedded into epoxy resin[2]. This leads to an increase of the section's thickness, while its area is compressed[3]. Compensation for compression is a necessity in the case of electron tomography, 3D reconstructions of the tissues from serial sections, and correct morphometric analysis of ultrastructures. For years several ways of correction existed: compression could be partially corrected by exposure of ultrathin sections to chloroform/acetone vapor (chemical correction)[2] or local heating of sections floating on top of hot water (thermal correction)[4]. Yet these classical ways of corrections are quite subjective and poorly reproducible. The new way of corrective action, emerging during the last decade, is the use of the oscillation diamond knife.

About a decade ago a study was accomplished that demonstrated the effect of an oscillating knife onto the section's compression. In that study, the authors used relatively soft objects (polymer polystyrene and Dinofagellates *Amphidinium carterae*) embedded in Lowicryl HM20 [1]. The degree of ultrasection compression was assessed by measuring the reduction of the cut area. Yet much harder objects, such as pollen grains, evaded investigation.

Here we choose pollen grains of *Aristolochia mandzhuriensis* as our object of study to research the effects of the oscillatory diamond knife. Angiosperm pollen grains possess a complex sporoderm which comprises of exine and intine. Exine contains a particular polymer sporopollenin and protects the male gametophyte against the adverse effects of the environment. Intine lacks sporopollenin and promotes pollen tube intrusion to achieve successful double fertilization. In the course of sporoderm development in pollen grains of *A.mandzhuriensis*, intine is formed by intensive exocytosis. This process is quite lengthy, during which long branched channels, which span the whole intine, appear. Spheroid pollen grains of *A.mandzhuriensis* are highly symmetrical. Their intine channels maintain an equal diameter throughout the entire perimeter of the pollen grain, providing an ideal model to study the section's deformation.

In the present study we examined the technique of ultrathin sectioning using the oscillating diamond knife and analyzed the ways of recompression of ultrastructures within hard biological samples (pollen grains) embedded in epoxy resin.

MATERIALS AND METHODS

SAMPLE PREPARATION

For transmission electron microscopy (TEM), pollen grains were embedded in epoxy-resin, according to standard protocol[5]. The Leica UC-5 Ultracut-R ultramicrotome (Leica Microsystems), equipped with an UltraSonic diamond knife (35°), was used for preparation of ultrathin sections (thickness ca. 50 nm, silver color, speed 0.6 mm/s) [1]. Sections were examined in the JEM-1011 transmission electron microscope (JEOL, Japan) at 80 kV accelerating voltage and captured with the CCD GATAN ES500W camera (Gatan) using Digital Micrograph software (Gatan).

The ultrathin sections were prepared using the oscillating diamond knife, from a single block, successively: first portion (no less than 5 sections) without the oscillation (stationary knife/ control), next portion (no less than 10 sections) with different oscillation frequency (US 20kHz, 27kHz and 40kHz) and final portion (no less than 5 sections) without the oscillation (stationary knife). Sections from this final portion were exposed to acetone vapor for 10 seconds immediately after sectioning. Four pollen grains were each cut with 20 and 40kHz oscillation frequency. Three pollen grains were cut with 27kHz oscillation frequency.

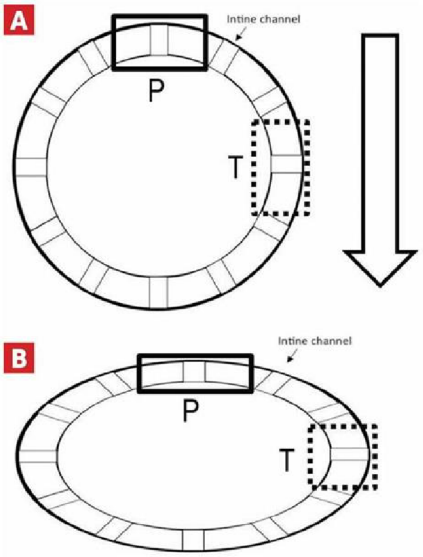


FIGURE 1 A schematic of intact (a) and deformed (b) pollen grains with intine channels marked with black arrows. White arrow shows the cutting direction. The bold line marks the location of the P-point micrograph; the dotted line marks the location of the T-point micrograph

The pollen grains that were cut through the nucleus (i.e. through the median part) were selected for the subsequent study. Micrographs of the intine of each pollen grain were taken at two points (P-point and T-point) (schematic on Fig. 1), using the same magnification of x100,000. The intine channels in P-point were oriented parallel to the course of the diamond knife, while in T-point they were oriented transversely to the course of the diamond knife. The intine channels diameters (ICD) were measured in both points (P and T), thereby 30-59 channel's measurements were done for each micrograph (1626 measurements in total).

STATISTICAL ANALYSIS

Statistical data processing was performed using the STATISTICA 7.1 software (http://www.statsoft.com). The data on ICD taken for each combination of factor levels (pollen grain, point (P or T), and frequency of the oscillation) were tested for normality using the Shapiro-Wilk test[6]. Two-factor analysis of variance was applied to evaluate the effects of the factors studied on the ICD.

Two-factor ANOVA plan: Dependent variable – ICD in pollen grains of

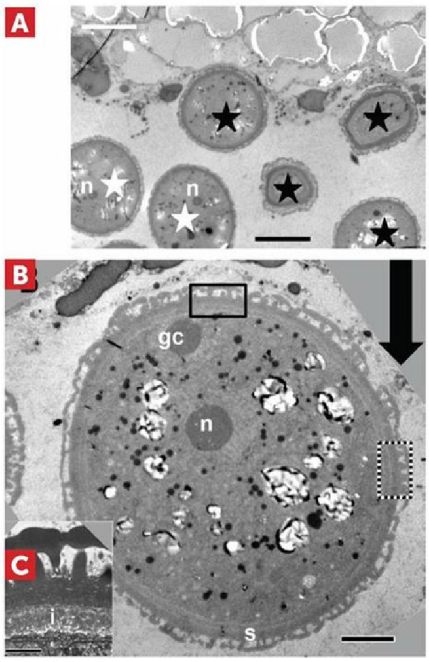


FIGURE 2, above, Ultrathin sectioning of A. mandzhuriensis pollen grains. (a) Pollen grains chosen for further investigation are marked with white stars, and disregarded pollen grains are marked wit black stars. Vegetative nucleus (n) is visible in the two pollen grains. (b) The medial section of a pollen grain. Vegetative nucleus (n) and generative cell (gc) are visible. The columellae (c) and intine (i) are marked in the sporoderm (s) (see inset). Black arrow shows the cutting direction. The black line marks the position of the P-point micrograph; the dotted line marks the position of the T-point micrograph. Bar, 5 um. Bar in the insert, 1 um.

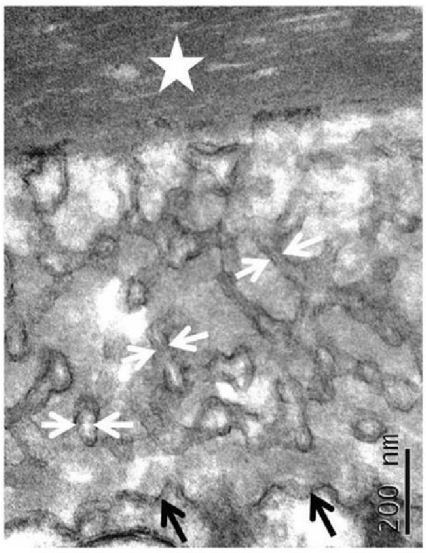
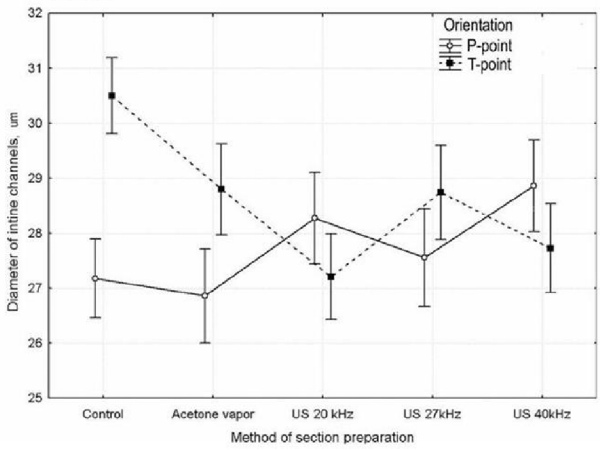


FIGURE 3 Intine channels. The lamellate endexine is marked with a white star; measured diameters of some channels in the intine are marked with white arrows, plasma-lemma is marked with black arrows. Bar, 200

FIGURE 4, above right, Mean diameters and their 95% confidence intervals for the variable 'ICD' in samples taken at a combination of factors 'Method of section preparation' and 'Orientation of intine part'. Factor levels are designated as in Table 2



Aristolochia mandzhuriensis (um); Factors (both fixed): F1) method of ultrathin section preparation; factor levels: no additional effects (control), exposure to acetone vapor, oscillation frequency (20, 27 ▯ 40 kHz); F2) orientation of intine part relative to the course of the knife; factor levels: P - parallel orientation, T - transversal orientation. Post-hoc comparison of means was accomplished by a modified test of Tukey for unequal groups (Unequal N HSD).

RESULTS

MORPHOLOGY OF THE ULTRATHIN SECTIONS

Serial sections were cut from the same block sequentially for each experiment. The difference between sections prepared with and without oscillation was obvious: sections that were prepared with oscillation were generally larger and lighter than those without oscillation (stationary knife). Each section was comprised of numerous pollen grains, cut though different levels (Fig. 2a).

The pollen grains we selected for analysis (marked with white stars in Fig. 2a) possessed visible nuclei, suggesting that their sporoderm was cut perpendicularly. Exine columelli, in this case, appear as columns (Fig. 2b, insert) in comparison to ovals on oblique sections.

The intine channels form a sparse network (Fig. 3). We measured the ICD in those points on the micrograph where the double membrane was clearly visible (arrows on Fig. 3). This ensures that the channel has been cut perpendicular to its membrane. So we can consider that the section passed close to the center of the channel, despite the orientations of measured channels being different (Fig. 3).

TEST FOR NORMALITY OF INTINE CHANNEL DIAMETER IN THE STUDIED SAMPLES

ICD in most of the samples were distributed normally (Appendix 1). From each of the seven samples whose distribution deviated from normal (marked in bold in Appendix 1), one or two extreme values were removed. After the removal of the outliers, the distribution in the samples approached normal. In total, eight values were removed from the complete dataset and its size became 1618. The error of measurements of structures on ultrathin sections, at magnification of x100,000, is ±6.5 nm.

TWO-FACTOR ANOVA

Results of two-factor ANOVA demonstrated that both factors studied (i.e. the method of ultrathin section preparation and the orientation relative to the knife's course) and their interaction significantly influences ICD (Table 1, below).

Effect	F-test	p-value
F1 - Method of section preparation	2.62	0.0332
F2 - Orientation of intine part, relative to the knife course (point P, point T)	10.63	0.0011
F1*F2	12.29	0.0000

MULTIPLE COMPARISONS OF MEANS (POST HOC TESTS)

The results of Post-hoc comparisons of means show that the average ICD at T-point (i. e. channels transversely oriented, according to the knife's course) for ultrathin sections, obtained without the oscillation (stationary knife), is significantly different both for the corresponding mean at P-point and for the means in the majority of other samples, obtained using acetone or oscillation (Table 2). When we compared means in two samples treated by acetone vapor, the difference between those for the P- and T-points was near-significant (p=0.0557, Table 2). The measurement error when using oscillation knife was ±8.0 nm.

DISCUSSION

During ultrathin section preparation, the mechanical impact of a diamond knife to the block deforms the sections and the ultrastructures embedded in the resin[2,3]. Here we designed a novel approach to statistically evaluate the deformation of ultrathin sections. For that we used highly spherical *Aristolochia mandzhuriensis* pollen grains, embedded into epoxy-resin and serially cut. The measurement of the intine channels diameters (ICD) on the pollen grain section in two perpendicular points around their sporoderm (Fig. 1) and comparing these to each other allowed to perform a direct estimation of geometric transformation of the section.

As we expected, without oscillation

F1 levels	F2 levels	1	1	2	2	20kHz	20kHz	27kHz	27kHz	40kHz
		P	T	P	T	P	T	P	T	P
1	P									
1	T	0.0000								
2	P	0.9999	0.0000							
2	T	0.1643	0.1176	0.0557						
20kHz	P	0.7209	0.0076	0.4054	0.9970					
20kHz	T	1.0000	0.0000	0.9999	0.1863	0.7548				
27kHz	P	0.9999	0.0002	0.9857	0.6372	0.9830	0.9999			
27kHz	T	0.2486	0.1178	0.0732	1.0000	0.9990	0.2763	0.6998		
40kHz	P	0.1342	0.1584	0.0415	1.0000	0.9932	0.1530	0.5698	1.0000	
40kHz	T	0.9949	0.0001	0.9269	0.7373	0.9964	0.9967	1.0000	0.8271	0.6784

TABLE 2 The results of post-hoc comparisons of means using the Unequal N HSD test (p-values). The test was applied to a dependent variable 'Diameter of intine channels' taken at a combination of factors 'Method of section preparation' (levels: 1 - no additional effects (control), 2 - exposure in acetone vapor, oscillation (US) 20kHz, US 27kHz, US 40kHz) and 'Orientation of intine' (levels: P-point: orientation of the intine channels parallel to the knife course, T–point: orientation of the intine channels transversely to the knife course)

(control) the stationary diamond knife strongly deformed the intine channels in the *A. mandzhuriensis* sections. The majority of the sub-cell structures, and especially intine channels, were compressed in the areas where the channels were directed parallel to the course of the knife (micrographs taken at point P) and flattened in the areas, where intine channels were oriented transversally (micrographs taken in point T). This resulted in a significant difference between ICD measurements in two indicated areas on the section of the same pollen grain, even though the direction of measured channels varied in the micrographs (Fig. 4). We hypothesized that, when the compression of the section will be corrected, one can expect the appropriate reduction of the difference between ICDs, measured at abovementioned orthogonal points.

In TEM practice, two ways of section decompression or dilatation are routinely used: by solvent vapor and by local heating[2,4]. The first method (dilatation in acetone vapor) was tested in the present study and ICDs were measured for four pollen grains. As expected, this approach didn't allow to completely neutralize the difference between ICD measurements in perpendicular P- and T-points (p=0.0557, Table 2). Moreover, this effect depends on a number of factors, like quality of epoxy resin and the environment; it is therefore difficult to forecast the effect and select the correct dose of the acetone vapor.

On the other hand, we detected a significant positive effect of the oscillation knife on *A. mandzhuriensis* ultrathin sections decompression. Apparently, the frequency of oscillation did not have a significant effect on decompression. Using three recommended oscillation frequencies (20, 27 and 40 kHz) we obtained statistically indistinguishable ICD measurements in the orthogonal points of the sporoderm (Table 2, Fig. 4), suggesting that pollen grains remain highly spherical upon sectioning. The oscillation implemented in oscillating diamond knife completely corrects the deformations of pollen grains structures on ultrathin sections. We concluded that our approach can successfully detect the statistically significant differences, and can be subsequently used for computation of section distortion.

SUMMARY AND CONCLUSIONS

Using the novel approach for evaluation of sections distortion we performed the comparison effects of oscillating and standard diamond knives onto the morphometric parameters of sub-cell structures in *A. mandzhuriensis* pollen grains. The minimal compression of the sections and subsequent minimal measurement errors were detected, when using the oscillating diamond knife. When using a standard diamond knife one should be aware that the morphometric parameters of the sub-cell structures strongly depend on their disposition relative to the cutting direction, and that structures, located perpendicular to the knife course, may be significantly distorted.

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BIOGRAPHY

Svetlana Polevova graduated from Lomonosov Moscow State University (MSU), biology faculty, Higher Plants department in 1989 and has a PhD degree awarded in 1999. Her PhD thesis is titled: *Morphology and Taxonomy of subgenera Heterolophus (Centaurea, Compositae)*. She has worked at the higher plants department of MSU since 1989. Her interests include palynology, micromorphology and electron microscopy.

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ABSTRACT

We designed a novel method for evaluating of the deformations in ultrathin sections induced by various cutting techniques. To achieve this we compared the diameters of intine channels measured on orthogonally oriented sections of *Aristolochia mandzhuriensis* sporoderm. The morphometric parameters of sub-cell structures strongly depend on their position relative to the direction to the knife course. According to our data, the oscillating knife allowed minimal deformation of sections, while the morphometric measurements were statistically significant and comparable. The oscillation frequency did not have a significant effect on section deformation.

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