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Original Research Paper

TISSUE VOLUME AND SECTION AREA CHANGES OF PARAFFIN AND METHACRYLATE EMBEDDED TESTICULAR SECTIONS: AN UNBIASED DESIGN STUDY

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ABSTRACT

For stereological estimates of structures in an organ such as total number (particles), length or surface area, the tissue change after processing (usually embedding of tissue blocks, sectioning of embedded blocks, and mounting and staining of sections) may need to be estimated to correct the results obtained on final sections so as to reflect the true results in the organ before processing. We proposed that the correction be made depending on the stereological principle used: correction for estimation with 3-dimensional and 2-dimensional measurements be based on different tissue volume and section area changes. Previous researches on tissue changes were limited and overlooked different corrections. Using paraffin and methacrylate embedded testicular sections, we studied, with an unbiased design, the tissue volume and section area changes after processing. The results demonstrated that the overall change of the actual tissue volume of paraffin sections was a marked shrinkage of 21%-23% while that of methacrylate sections was a small expansion almost negligible; compared with area of the embedded block face, the final section area had a 4% decrease in paraffin but an 8% increase in methacrylate. The results were tentatively used for correction of estimates in some of previous studies. The present study provides both methods and data for experiments involving the study of tissue change for better stereological estimation.

Keywords: methacrylate sections, paraffin sections, section thickness, stereology, tissue shrinkage.

INTRODUCTION

Stereological Background

It is marvelous, which many histopathological researchers must have been aware of for half a century, that microscopic structures can be quantitatively studied with mathematics- and statistics-based stereological methods, including computer assisted stereological tools (Weibel, 1979 & 1980; Gundersen *et al.*, 1988a & 1988b; Yang *et al.*, 1990; Zhengwei *et al.*, 1997; Nyengaard, 1999; Yang, 2012). The principle is simple. Just by means of counting (on fields of view sampled on tissue sections cut from an biological organ), for instance, we can estimate X_V: the amount (e.g. volume, surface area, length or number) of any structures (including linear structures or discrete particles such as nuclei) per volume of (some part of the) organ (the so-called reference space that contains the structures concerned). Then by multiplying the density (X_V) by the volume of the organ (the reference volume V), we can estimate the total amount of the structures (X) in the organ:

$$X = X_V \times V \quad (1)$$

This parameter of total amount, most valuable and commonly obtained in stereological practice, reflects the true, absolute amount of structures in the organ, unaffected by the organ size, and can thus best reflect (i) the overall function of the structures in the organ and (ii) the quantitative changes of the structures between different experimental conditions or groups, avoiding the serious problem of reference trap (Braendgaard & Gundersen, 1986; Nyengaard, 1999; Yang, 2012).

That being said, many researchers may have been confused about or tended to ignore what the organ volume (V) should be and what the total amount (X) obtained actually represents. In practice, we (should) usually obtain the volume of the fresh organ before tissue processing (usually slicing & embedding of tissue blocks, sectioning of embedded blocks, mounting of sections, and staining & coverslipping of mounted sections), the process of obtaining final sections (from the organ) ready for microscopic observation and

measurement. This volume, designated as *V(pre)*, can be readily obtained by using methods such as water displacement (for large organs) or one (for small or large organs) based on weight and specific gravity (density) of the organ (Weibel, 1979; Gundersen *et al.*, 1988a; Yang, 2012; Guo *et al.*, 2019). However, stereological measurements are performed on final sections, i.e. the structural density (X_V) is obtained after processing. So what we first estimate is in fact the total structural amount after processing:

$$X(post) = X_V \times V(post) \quad (2)$$

where *V(post)* is the organ volume after processing, which is, unlike *V(pre)*, somewhat difficult to understand and estimate.

V(post) can be imagined as volume of the processed organ, reconstructed from all the serial sections that can be cut from the whole fresh organ, and *X(post)* is the total amount of the structures in the processed organ. Take the estimation of particle number as an example,

$$N(pre) = N(post) = N_V \times V(post) \quad (3)$$

where (i) *N(pre)* and *N(post*) are the total numbers of particles in the organ before and after processing, respectively, the two numbers being equal assuming that processing does not affect the number of particles, and (ii) N_V is the numerical density of particles obtained after processing.

V(post) is usually estimated by the Cavalieri's principle (Gundersen *et al.*, 1988a; Yang, 2012). (i) When the organ concerned is completely cut into serial sections, we have a direct estimate of *V(post)* with the following Cavalier's estimator:

$$V(post) = t \times \Sigma a \quad (4)$$

where *t* is the thickness of serial sections and $\sum a$ is the total area of all the serial sections. Sample some of the tissue blocks from the organ and sample some of the serial sections from the blocks in a systematic (uniform) random manner (with known sampling fractions), we can also estimate ($t \times \Sigma a$) according to the fractionator principle, without necessarily cutting the organ all into serial sections (Gundersen *et al.*, 1988b; Yang, 2012; Sadeghinezhad & Nyengaard, 2019), see Equation 19 below. (ii) In most cases, only some random, single sections are obtained from the organ for stereological study and the organ's *V(post)* cannot be directly estimated from the sections. In many cases, we need to perform a separate study to estimate the ratio of tissue volume change (*RVC*), thus indirectly estimating *V(post)*:

$$V(post) = V(pre) \times RVC \quad (5)$$

where *RVC* can be estimated by sampling some tissue blocks from an organ, preparing serial sections and estimating the organ volumes before and after processing (as we do in Part "First Experiment: Study of Tissue Volume Change" below):

$$RVC = \frac{V(post)}{V(pre)} \quad (6)$$

Note that the *V(post)* in Equation 3 (for estimation of total particle number) can be directly estimated by the Cavalier's principle (Equation 4), or indirectly estimated using Equations 5 & 6 that also involve the Cavalier's principle (Equation 4). Now comes a tricky part: the section thickness (*t*) in Equation 4 should be considered in two ways depending on the stereological principle used for the number estimation. (i) When the number is estimated with the physical or optical disector, i.e. the particles are counted in 3D (3-dimentional) space (Gundersen *et al.*, 1988b; Nyengaard, 1999; Yang, 2012; Xu *et al.*, 2019), the section thickness should be the actual thickness – *t(act)* – measured thickness of the sections after processing irrespective of whether the processing leads to uniform or non-uniform shrinkage or expansion (Fig. 1). That is, the *V(post)* in Equation 3 should be the actual *V(post)*:

$$V(post, act) = t(act) \times \Sigma a \quad (7)$$

where *t(act)* is best measured directly using an electronic microcator while the section is being observed with an oil immersion lens (Dorph-Petersen *et al.*, 2001; Xiang & Yang, 2014). (ii) When the number estimation is based on 2D (2-dimensional) counts of particle profiles on sectional planes (Weibel, 1979; Yang *et al.*, 1990), however, the section thickness should be the virtual thickness – *t(vir)* – thickness of the section after uniform change in proportion to the section area change [Figs. 1(2) & 1(4)]. In this case, the *V(post)* in Equation 3 should the virtual *V(post)*:

$$V(post, vir) = t(vir) \times \Sigma a \quad (8)$$

The *t(vir)* can be estimated by,

$$t(vir) = t(ba) \times RAC^{\frac{1}{2}} \quad (9)$$

where *t(ba)* is the block advance (i.e. thickness set by the microtome) and *RAC* is the ratio of section area change. *RAC* can be estimated by,

$$RAC = \frac{a(post)}{a(eb)} \quad (10)$$

where *a(post)* is the area of the final section after processing and *a(eb*) is the area of the embedded block face (cut-surface) from which the section is cut, see Part "Second Experiment: Study of Section Area Change" below. Of note, where serial sections are used for

volume estimation (Equations 4, 7 and 8), a separate study is usually required for *RAC* estimation (Equation 10) since it is practically impossible to estimate both *a(post)* and *a(eb)* while cutting serial sections.

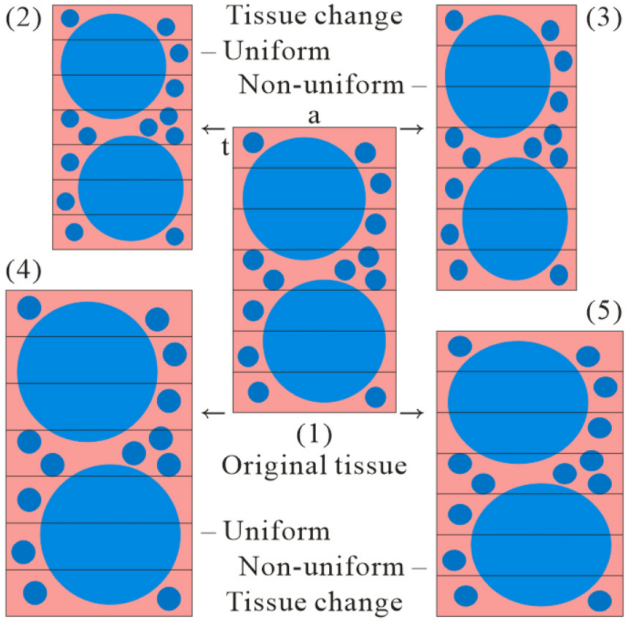

Fig. 1. *Schematic illustration of tissue change: from original tissue before processing (1) to tissue after processing (2-5). The original tissue shown is a projection of some cuboid-shaped tissue (pink) containing large and small spherical particles (blue). The tissue is divided into "sections", each with an upper surface (length and width unseen) area of "a" and a thickness of "t". (2) & (4): uniform change in 3 dimensions (length, width and thickness); (3) & (5): non-uniform change. Of note, the thickness change is proportional to the area change in (2) & (4), not in (3) & (5).*

Of note, (i) the area in comparison with *a(post)* in Equation 10 is the area of the tissue block face after embedding, not the area of the fresh tissue block face before embedding, (ii) the block advance is the thickness (of sectioning the embedded tissue) set at the step of sectioning, and (iii) the *RAC* in Equation 9 is used to consider the virtual thickness change in proportion to the section area change after sectioning, for the estimation of the virtual *V(post)* (see Equations 8, 9 and 10).

The virtual *V(post*) obtained by Equations 8 -10 can be imagined as the volume of the embedded organ after a 3D uniform magnification or minification of the organ based on the scale of the sectional area (2D) change, like a 3D uniform zooming with a computer mouse, or as the organ space consisting of serial sectional planes, with the interval between planes being the virtual thickness.

Since we have *V(post, act)* and *V(post, vir)*, we also have a block advance based *V(post)*:

$$V(post, ba) = t(ba) \times \Sigma a \quad (11)$$

Based on Equations 6 and 11, we have a block advance based ratio of tissue volume change:

$$RVC(ba) = \frac{V(post,ba)}{V(pre)} \quad (12)$$

Then we can estimate the actual or virtual ratio of tissue volume change based on the ratio of the actual or virtual section thickness change by,

$$RVC(act) = \frac{V(post,act)}{V(pre)} = RVC(ba) \times \frac{t(act)}{t(ba)} \quad (13)$$

$$RVC(vir) = \frac{V(post,vir)}{V(pre)} = RVC(ba) \times \frac{t(vir)}{t(ba)} \quad (14)$$

Note, from Equation 13, we have,

$$\frac{RVC(act)}{RVC(ba)} = \frac{t(act)}{t(ba)} \quad (15)$$

And from Equations 14 and 9, we have,

$$\frac{RVC(vir)}{RVC(ba)} = \frac{t(vir)}{t(ba)} = RAC^{\frac{1}{2}} \quad (16)$$

To summarize in a practical way, (i) for total particle number estimation based on particle counts in 3D space (using 3D probes - physical or optical disectors), we have:

$$N(pre) = N(post) = N_V \times V(pre) \times RVC(act) \quad (17)$$

And (ii) for total particle number estimation based on 2D counts (number of particle profiles per area of section), we have:

$$N(pre) = N(post) = N_V \times V(pre) \times RVC(vir) \quad (18)$$

where *RVC(act)* and *RVC(vir)* can be estimated in a separate, comparable study (Equations 13 & 14).

In essence, whether for global estimation (estimation of total amounts) of particle numbers or other geometric properties, the *V(post)* in Equation 2 should be (i) the actual reference volume reconstructed from all the serial sections with actual thickness when the stereological estimation is based on measurement in 3D space - measurement results per volume of reference space, or (ii) the virtual reference volume reconstructed from all the serial sections with virtual thickness when the stereological estimation is based on measurement on 2D plane - measurement results per area of section. The virtual reference volume is proportional to the section area and the virtual (not actual) section thickness, and the virtual section thickness is proportional to the block advance and the section area change (Equation 9), unrelated to the actual section thickness.

Note that, unlike total particle number estimation, assumption of uniform tissue volume change after processing [Figs. 1(2) & 1(4)] must be accepted, although it may not be valid in practice (Dorph-Petersen *et al.*, 2001; Zhao *et al.*, 2010), for total volume, surface area or length estimation, otherwise we cannot determine, with observation and measurement on final sections, what has happened, during processing, to the structural amounts relative to the reference volume. Under the prerequisite of uniform change, (i) the volume fraction (volume of structures per volume of organ) estimated after processing can be taken as the volume fraction before processing, so we can obtain the total volume of any structures before processing by: (the volume fraction estimated after processing) × $V(pre)$. (ii) The total surface area of any structures before processing can be obtained by: (the surface area density estimated after processing) × $V(pre)$ × $RVC(vir)^{1/3}$; the total length of linear structures before processing can be obtained by: (the length density estimated after processing) × $V(pre)$ × $RVC(vir)^{2/3}$ (Yang, 2012).

Study of Tissue Volume and Section Area Changes after Processing

We perform stereological measurements on final sections that are originally cut from organs and then subjected to histopathological processing, a series of procedures such as tissue fixation & dehydration, tissue blocks' sampling, embedding & sectioning, and tissue sections' mounting, staining & coverslipping (Dorph-Petersen *et al.*, 2001). The processing may vary from researcher to researcher, from laboratory to laboratory, and from study to study, likely leading to variable tissue volume change and thus introducing variable systematic error. So it is better to correct the error, if the correction can be made by studying the changes properly and reliably, so that the corrected data can best represent the true value in the original organ.

The core part of correction for the tissue volume change is to estimate $V(post)$, the volume of organ (reference space) after processing. $V(post)$ is mostly estimated by the Cavalieri's principle (Equation 4). Rarely was it pointed out, however, that this volume estimation should be considered in two ways (see Part "Stereological Background"). In one way, $V(post)$ should be the actual reference volume, i.e. the actual measured thickness of sections should be used for the Cavalieri's volume estimation. The volume estimated in this way is essential for total number estimation with the disector based on Equations 3, with the exception of total number estimation by the fractionator principle (Gundersen *et al.*, 1988b; Nyengaard, 1999; Dorph-Petersen *et al.*, 2001; Yang, 2012). In the other way, $V(post)$ should be the virtual reference volume, i.e. the section thickness for the Cavalieri's volume estimation (Equation 4) should be the virtual thickness, one that is calculated from the block advance (thickness set by the microtome) and the ratio of section area change (Equation 9). The virtual reference volume is used for correction of global estimates (e.g. total surface area, length or number per organ) based on 2D measurement, and for that of local size estimates (e.g. mean size of particles) based on 2D measurement as well, which is beyond the scope of the present paper.

In previous studies dealing with the volume change, few actually estimated the $V(post)$ with consideration of the actual thickness of sections (best measured with a microcator). Many studies used tissue blocks for the volume change study and the linear (length or width) change of the blocks was used to reflect the volume change, with concerns over the accuracy of the blocks' volume estimation. In previous studies dealing with the section area change, few actually measured the areas at different stages of processing. Concerns also include clear viewing and confident measurement of the block faces and sections.

For examples, researcher ZWY (and co-authors), whose major interest of research is stereological study of reproductive organs using paraffin and resin sections with light microscopy, previously studied the tissue shrinkage of paraffin embedded (Yang & Cui, 1989) and Epon-Araldite embedded (Yang *et al.*, 1990) testicular sections, but neglected to study the actual section thickness (Yang & Cui, 1989) and the section area change or virtual section thickness (Yang *et al.*, 1990). Then the tissue shrinkage of methacrylate testicular sections was studied, neglecting to study the actual section thickness as well (Zhengwei *et al.*, 1997); effects of fixation and dehydration on organ sizes were studied and paraffin and methacrylate sections were compared in a stereological study, both being unable to reflect the final tissue volume change (Zhao *et al.*, 2010). Thereafter attention was given to study of the section area change of paraffin testicular sections by using scan images (Xiang *et al.*, 2018), but the area of fresh tissue block faces was not studied, and methacrylate sections were not included in the study due to difficulty in clear viewing of the methacrylate block faces and sections.

Therefore, the present experiment was carefully designed to study both the tissue volume change and the section area change of testicular sections. Specifically, testicular tissue blocks from rats of different ages or blocks of different sizes and shapes were compared; both paraffin and methacrylate sections were used; the volume of testis after processing was unbiasedly

determined by the Cavalieri's principle in combination with the fractionator principle; methods of observing block faces and sections were improved; the actual thickness of sections after processing were directly measured; and areas of the block faces and sections observed at all major stages of tissue processing were unbiasedly determined with the test-point counting method. With such a comprehensive, unbiased design, the present study aimed not only to clarify the testicular tissue change after processing but also provide both methods and data for experiments involving the study of tissue change for better stereological estimation.

MATERIALS AND METHODS

First Experiment: Study of Tissue Volume Change

Three groups of 6 testes of different sizes from three groups of normal male Sprague–Dawley rats aged 1 month (pubertal), 3 months (mature) and 5 months (adult), respectively, were obtained from the Animal Center of North Sichuan Medical College. After anaesthesia with intraperitoneal injection of pentobarbital sodium (50 mg/kg), one testis with its surrounding tissues was immediately removed from one rat and immersion fixed in Bouin's fluid. Two days later, the organs were stored in 70% ethanol, for a few months before further experiment. The samples were from an animal study by Guo *et al.* (2019) which was approved by the Ethics Committee of North Sichuan Medical College (CBY13-A-ZP05).

Sampling and embedding of tissue blocks

Dissected from the surrounding tissues, each testis with a complete capsule (tunica albuginea) was weighed on an electronic balance (accuracy 0.1 mg) and its density (around 0.925 g/cm^3) was determined in graded ethanol solutions of known densities (Yang, 2012; Guo *et al.*, 2019).

Each small testis (pubertal), with a height of about 10 mm, was cut into 4 circular slices (blocks, thickness approximately equal) perpendicular to its long axis (height). Two alternate blocks, determined in a random manner, were used for paraffin embedding and the other 2 blocks for methacrylate embedding. That is, the sampling fraction of tissue blocks, SF(blo), for either paraffin or methacrylate embedding was 1/2 (Table 1).

Table 1: *Tissue volume change related results obtained from the First Experiment*

	Paraffin embedding			**Methacrylate embedding**		
	Small testes	**Medium testes**	**Large testes**	**Small testes**	**Medium testes**	**Large testes**
Volume (cm^3) of fresh testis[a]	0.178 (17.1%)	1.387 (8.1%)	1.556 (13.9 %)	0.178 (17.1%)	1.387 (8.1%)	1.556 (13.9 %)
[a] V(pre), volume of the testis before processing, estimated by weight and density of the fresh (intact) testis						
Sampling fraction of blocks[b]	0.500	0.167	0.063	0.500	0.167	0.063
[b] Fraction of the testicular tissue blocks sampled from the fresh testis (= Number of blocks sampled / Total number of blocks cut)						
Sampling fraction of sections[c]	0.067	0.067	0.067	0.100	0.100	0.100
[c] Fraction of the testicular sections sampled systematically from all serial sections cut from the testicular block						
Thickness (μm) of sections[d]	14.6 (0.9%)	14.6 (4.5%)	14.5 (1.8%)	20.7 (4.0%)	21.8 (2.9%)	21.1 (2.4%)
[d] Actual thickness, t(act), of testicular sections after staining. [The thickness set by microtome: 14 (paraffin) and 25 (methacrylate).]						
Mean area (mm^2) of sections[e]	12.8 (16.4%)	23.1 (7.6%)	13.2 (17.1%)	17.9 (12.7%)	33.5 (15.0%)	19.4 (16.8%)
[e] Average area of testicular sections (stained). [Average numbers of sections: 25, 35, 26, 24, 32 and 25, respectively, from left to right.]						
Volume (cm^3) of processed testis[f]	0.142 (23.8%)	1.061 (9.5%)	1.206 (27.4%)	0.178 (15.2%)	1.399 (10.9%)	1.603 (18.5%)
[f] V(post, act), actual volume of the testis after processing, estimated by the Cavalieri's principle (see Equation 7 in the text)						
RVC(act)[g]	0.793 (12.0%)	0.766 (7.7%)	0.771 (18.6%)	1.005 (10.8%)	1.009 (6.8%)	1.034 (14.3%)
[g] Actual ratio of testicular tissue volume change, equal to [V(post, act) / V(pre)], see Equation 13 in the text						
RVC(ba)[h]	0.758 (11.7%)	0.732 (6.0%)	0.746 (18.0%)	1.213 (8.8%)	1.158 (8.8%)	1.227 (14.5%)
[h] Block advance based ratio of testicular tissue volume change, see Equation 12 in the text						
RVC(vir)[i]	0.760 (11.7%)	0.716 (6.0%)	0.730 (18.0%)	1.262 (8.8%)	1.221 (8.8%)	1.294 (14.5%)
[i] Virtual ratio of testicular tissue volume change, see Equation 14 in the text*						

Data shown as "x (%)": "mean (coefficient of variation)" calculated from each sub-group (n=6). Three groups of testes (small, medium-sized and large) were obtained from three groups (pubertal, mature and adult) of normal Sprague-Dawley rats (6 rats per group, with one testis being sampled from each rat). Four testicular tissue blocks were cut and sampled in a systematic random manner from each testis, with 2 systematic blocks being embedded in paraffin (sub-group) and the other 2 blocks in methacrylate (another sub-group). *The ratios of testicular section area change (*RAC*s) necessary for the RVC(vir) estimation (see Equations 9, 10 & 14 in the text) were borrowed from Table 2, being 1.005, 0.957, 0.957, 1.083, 1.112 and 1.112, respectively, in the 6 sub-groups from left to right.

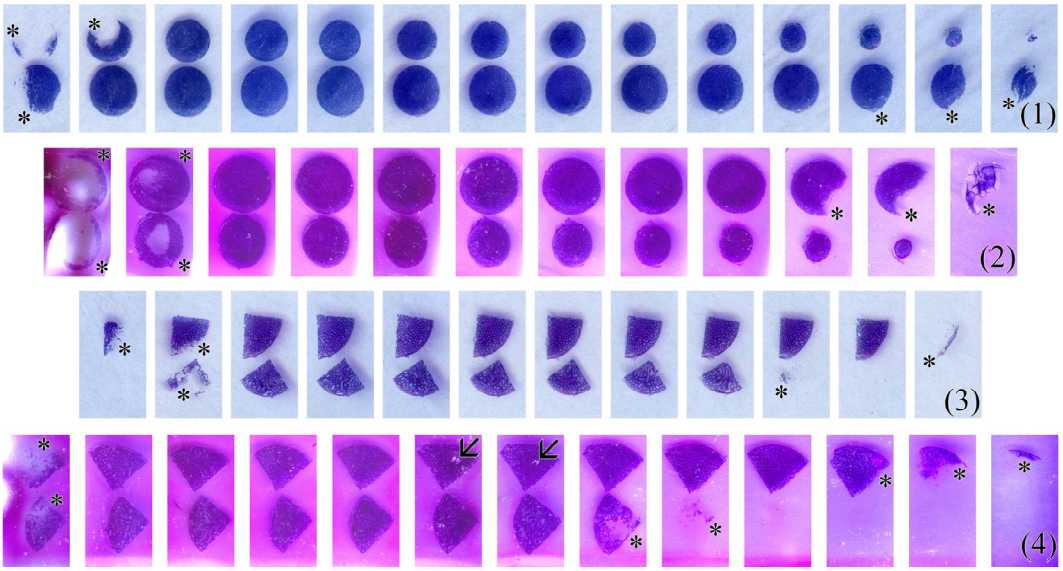

Fig. 2. *Serial sections of paraffin (1 & 3) and methacrylate (2 & 4) embedded testicular tissue blocks. Each embedded block contains two tissue blocks that were cut and sampled from one testis; the testes were obtained from normal male Sprague-Dawley rats aged one-month (1 & 2) or five-months (3 & 4). The tissue block is a circular slice of tissue (1 & 2) or a quarter of the slice (3 & 4) that was cut in a direction perpendicular to the testicular long axis. One out of every fifteen serial paraffin sections (thickness set by microtome 14 µm), or one out of every ten serial methacrylate sections (thickness set by microtome 25 µm), was sampled and stained with periodic acid-Schiff's reagent and hematoxylin, with the embedding medium being removed (paraffin) or not removed (methacrylate) during staining. The section image was scanned from the coverslipped section (stained) on glass slide, each (shown here) with an image processing (Photoshop) of automatic contrast (paraffin), or automatic contrast followed by an increase (+28) of brightness and an increase (+28) of contrast (methacrylate). *, non-intact tissue sections cut from the two ends of the tissue block; ↙, an empty area (artifact) inside the tissue section. The width and height of each image (small rectangular panel in the figure): 7.5 and 14.5 mm.*

Each medium-sized testis (mature), with a height of about 21 mm, was first cut into 6 circular slices (thickness approximately equal) perpendicular to its long axis. Two slices were sampled in a systematic random manner (Gundersen *et al.*, 1988b; Nyengaard, 1999; Dorph-Petersen *et al.*, 2001; Yang, 2012): the 1st & 4th, the 2nd & 5th, and the 3rd & 6th slices were sampled in turn from different testes. Each of the 2 slices sampled was then cut into 2 halves (semicircular blocks), with one block (randomly chosen) being used for paraffin and the other one for methacrylate. So the SF(blo) for either paraffin or methacrylate was 1/(3×2) (Table 1).

Each large testis (adult), with a height of about 22 mm, was first cut into 8 circular slices (thickness approximately equal) perpendicular to its long axis. Two slices were sampled in a systematic random manner. Each of the 2 sampled slices was then cut into 4 quadrants (blocks), with one block (randomly chosen) being used for paraffin and another one (randomly chosen as well) for methacrylate. The SF(blo) for either paraffin or methacrylate was therefore 1/(4×4) (Table 1).

The 2 tissue blocks obtained from each testis were embedded as one embedded block for either paraffin or methacrylate embedding (Fig. 2).

For paraffin embedding of the tissue blocks, the wax used was Paraplast by Leica Biosystems Richmond (USA), melting point 56ºC. The basic embedding procedures we worked out were dehydration in 100% ethanol for 3 × 1 hours (i.e. 1 hour for 3 changes), clearing in 1-butanol for 1 hour plus 2 × 2 hours, infiltration first in butanol and melted paraffin (1:1) for 40 minutes and then in melted paraffin for 2 × 80 minutes in a container (60ºC), and finally embedded in a mould on a cooling table at room temperature.

For methacrylate embedding, the resin used was a glycol methacrylate (2-hydroxyethyl methacrylate), Historesin by Leica Microsystems Nussloch GmbH (Germany). The embedding procedures, which, essentially based on the manufacturer's instruction, we have been following for years (Zhengwei *et al.*, 1997; Zhang *et al.*, 2002), were dehydration in 100% ethanol for 3 × 1 hours, clearing in 1-butanol for 3 hours, infiltration in the infiltration solution [100 mL basic resin

(methacrylate) plus 1 g activator containing dibenzoylperoxide] overnight at 4ºC, and embedding (polymerization) in the embedding solution (15 mL infiltration solution plus 1 mL hardener containing dimethyl sulfoxide) in a mould at room temperature.

Sectioning, sampling and staining of tissue sections

Attached to a rectangular plastic piece (supporting block) that was clamped to microtome, each embedded tissue block was serially and completely sectioned using the same semi-automatic microtome (RM2235, Leica Biosystems Nussloch GmbH, Germany). The knives for cutting paraffin sections were Leica high profile microtome blades and those for methacrylate sections were Leica tungsten carbide knives; the section thickness set by the microtome [*t(ba)]* was 14 μm for paraffin and 25 μm for methacrylate (Xu *et al.*, 2019).

One out of every 15 serial paraffin sections or 10 serial methacrylate sections was sampled in a systematic random manner and stained for area measurement. Occasionally, the sampled section was of poor quality and a nearest neighbor section was used instead. So the sampling fraction of tissue sections, SF(sec), was 1/15 for paraffin and 1/10 for methacrylate (Table 1).

All sections underwent procedures, including staining with periodic acid-Schiff's reagent and hematoxylin, with a similar protocol we have been following for years (Zhang *et al.* 2002; Xiang & Yang, 2014; Guo *et al.*, 2016; Xiang *et al.*, 2018; Xu *et al.*, 2019). Briefly, (i) each section was floated onto an adhesive glass slide (with positively charged surface) from a distilled water bath, at 37°C for paraffin sections and at room temperatures for methacrylate sections, and was left dried up: paraffin sections, placed vertically in a slides box at room temperature; methacrylate sections, placed flat on a hotplate at 80°C for a few minutes. (ii) Key staining steps for paraffin sections included: heating of sections in an incubator at 50°C (1 hour) for prevention of section detachment; dewaxing in xylene (2×3 minutes); staining in 1% periodic acid (15 minutes), Schiff's reagent (20 minutes) and hematoxylin (90 seconds); dehydration in 70% ethanol (2 minutes), 95% ethanol (2 minutes) and absolute ethanol (2×2 minutes); and clearing in xylene (2×2 minutes). Key staining steps for methacrylate sections included: heating of sections on a hotplate at 90°C (30 minutes) for prevention of section detachment; staining in 1% periodic acid (30 minutes), Schiff's reagent (50 minutes) and hematoxylin (30 minutes); dehydration in 95% ethanol (2 minutes) and absolute ethanol (2×2 minutes); and clearing in xylene (2×2 minutes). (iii) All sections after staining were mounted with a neutral balsam (refractive index ~1.52). Unlike paraffin sections, methacrylate sections did not have the embedding medium (plastic) dissolved or removed in all procedures.

Estimation of total section area

Each of the testicular sections sampled and stained (21-36 paraffin and 22-37 methacrylate sections per testis) was observed on a computer screen through a × 1.25 objective lens (PlanApo N, numerical aperture 0.04) of an Olympus BX53 microscope equipped with a stereology system (newCAST, Visiopharm, Denmark). Evenly spaced, rectangularly distributed test-points (centers of cross-shaped lines) covering the whole section were generated and superimposed on screen. The area that each point occupied or was associated with, equal to the product of the horizontal and vertical distances between points along the X-axis and Y-axis respectively, was 0.479 mm^2 for smaller serial sections and 0.767 mm^2 for larger serial sections. The total area of the serial paraffin or methacrylate sections from each testis was estimated by multiplying the area associated with each point by the total number of points hitting the sections, the total number being 499-1120 (paraffin) or 513-1693 (methacrylate).

Measurement of actual section thickness

From the serial testicular sections for area measurement (above), 3-4 paraffin and 6-8 methacrylate sections per testis were re-sampled in a systematic random manner for thickness measurement. The sections were observed and measured on a computer screen through a × 100 immersion oil lens (PlanApo N, numerical aperture 1.40) of an Olympus BX51 microscope equipped with a stereology system (newCAST, Visiopharm, Denmark), the refractive index of the immersion oil used (Olympus) being 1.518. Fields of view were sampled on the sections in a systematic random manner (meander sampling) by means of a computer-assisted motorized stage (ProScan III, Prior Scientific Inc., USA); the fraction (in area) of fields sampled on each set of serial sections remained unchanged during measurement, set at 0.35%-0.90% (paraffin) or 0.25%-0.45% (methacrylate). A frame (35 μm × 26 μm) was generated and superimposed at the center of each field. The height of the testicular tissue in the frame, as measured between the top and bottom surfaces of the tissue section with an electronic microcator (Dr. Johannes Heidenhain GmbH, Germany; maximum position error: ± 0.13 μm within a distance of 12 mm), was taken as the actual section thickness at the field (Xiang & Yang, 2014; Xu *et al.*, 2019). Of note, the top or bottom surface was determined, in serially focusing up and down through the

section, as the focal plane where the tissue structure (especially stained granules of nuclear chromatin) just began to appear or disappear. The average of the thicknesses measured was the mean actual thickness of the serial sections and the number of thicknesses measured was 21-38 (paraffin) or 24-58 (methacrylate) for each set of serial sections, with the average within-individual (set) CVs (coefficients of variation, for groups of testes shown in Table 1) being 2.7%-4.7% and 7.4%-11.9% for paraffin and methacrylate sections, respectively.

Ratio of tissue volume change

The volume of the testis before processing, *V(pre)*, was directly calculated, equal to its weight divided by density. According to the Cavalieri's principle (Equation 4) and the fractionator principle, the actual volume of the testis after processing (paraffin or methacrylate) was estimated by,

$$V(post, act) = \left[\frac{1}{SF(blo)}\right] \times \left[\frac{1}{SF(sec)}\right] \times \Sigma a \times t(act) \qquad (19)$$

Dividing the *V(post, act)* by *V(pre)*, we obtained the actual ratio of the testicular tissue volume change, *RVC(act)*, for each sub-group (Equation 13 and Table 1). Replacing the actual section thickness with the block advance or virtual section thickness - *t(ba)* or *t(vir)* - in the above calculations, we obtained the block advance based ratio or the virtual ratio of the testicular tissue volume change, *RVC(ba)* or *RVC(vir)*, for each sub-group (Equations 11, 12 & 14 and Table 1). Of note, the ratios of the section area change (*RAC*s) that were needed in the estimation of the virtual section thickness (Equation 9) were from the Second Experiment (below) with similar testicular sizes and block shapes (Part "Second Experiment: Study of Section Area Change" and Table 2).

Error analysis

The total sampling error of the above stereological estimation of V(post) with Equation 19 arose mainly from the sampling of tissue blocks (i.e. estimation of the testis volume from the volume of the sampled blocks according to the fractionator principle) and the Cavalieri's estimation of the blocks' volume. The error of blocks' sampling, which people might be unconcerned with, was tentatively analyzed by the weights of tissue blocks. After the 2 tissue blocks for paraffin and the 2 tissue blocks for methacrylate were cut (systematically sampled) from each testis (Part "Sampling and embedding of tissue blocks"), the tissue blocks and all other tissue blocks cut in the same way from the testis were weighed to obtain the weight fraction of the 2 tissue blocks (for paraffin or methacrylate), which should approximate the sampling fraction [SF(blo)] of the 2 tissue blocks (Part "Sampling and embedding of tissue blocks") assuming homogeneous specific gravity of the tissue blocks. The error of Cavalieri's estimation, which people might be more concerned with, was analyzed as previously described (Yang *et al.*, 2000), without considering the error which the actual section thickness measurement might contribute. Briefly, each glass slide, on which there was 1 or 2 tissue sections (Fig. 2), was regarded as "one section". In this way, the sample of serial sections was divided into 2 systematic sub-samples, one including the first, third, … "sections" (odd numbers) while the other including the second, fourth, … "sections" (even numbers). Summating the total numbers of test-points counted (Part "Estimation of total section area") on the sections of the 2 sub-samples, respectively, we had an approximate coefficient of error (CE) for the V(post) estimation:

$$CE = \left(\frac{1}{2}\right)^{\frac{1}{2}} \times \frac{|x_1 - x_2|}{(x_1 + x_2)} \qquad (20)$$

where x_1 & x_2 are the total point numbers obtained from the 2 sub-samples, respectively.

To determine if there was considerable systematic (machine) error in the section thickness set by the microtome [*t(ba)* in Part "Sectioning, sampling and staining of tissue sections"], another 18 testicular blocks embedded in methacrylate, obtained from some other studies and prepared in the same way (Part "Sampling and embedding of tissue blocks"), were serially cut using the same microtome at thickness 25 μm [*t(ba)*] and this thickness was estimated by dividing the difference of the block heights, measured before and after serial sectioning with a digital vernier caliper (Shanghai Meinaite Industrial Company Ltd., China; accuracy 10 μm), by the total number (approximately 100-200) of serial sections cut (Xiang *et al.*, 2018). Of note, to minimize error of this thickness estimation, the block heights were measured from a corner (edge) of the methacrylate block and the first section cutting the corner was taken as the first of the serial sections.

Second Experiment: Study of Section Area Change

Two groups of testes (12 per group) from 2 groups of normal pubertal and mature male Sprague–Dawley rats (6 per group) were obtained from the Animal Center of North Sichuan Medical College, the average testicular volume being 0.38 cm3 (CV, i.e. coefficient of variation, 4.8%) for the smaller testes and 1.30 cm3 (6.7%) for the larger testes. After fixation in the same way as described above, the testes were stored in 70% ethanol for 2 weeks before further experiment.

Table 2: *Section area and diameter related results obtained from the Second Experiment.*

	Paraffin embedding		Methacrylate embedding	
	O-blocks (n=6)	C-blocks (n=6)	O-blocks (n=6)	C-blocks (n=6)
Study of smaller testes				
Area of FBs [a] (mm^2)	38.9 (3.7%)	19.2 (14.8%)	40.6 (3.4%)	22.3 (8.5%)
Ratio [r] of EB [a] to FB areas	0.887 (3.4%) [f,o]	0.927 (6.7%) [f,c]	1.052 (2.9%) [f]	1.078 (3.8%) [f]
Ratio [r] of US [a] to EB areas	1.006 (3.8%) [f,o]	0.968 (7.1%) [f,c]	1.119 (5.5%) [f,e]	1.129 (4.8%) [f,e]
Ratio [r] of SS [a] to EB areas (i.e. *RAC*)	1.005 (3.1%) [f,o]	0.952 (6.4%) [f,c]	1.083 (4.7%) [f,e]	1.098 (3.9%) [f,e]
Mean diameter of FBs [d] (mm)	7.2 (1.5%)	5.9 (6.9%)	7.2 (1.2%)	5.8 (5.6%)
Ratio [r] of EB [d] to FB mean diameters	0.946 (1.5%) [f,o]	0.944 (2.3%) [f,c]	1.017 (1.1%) [f]	1.028 (2.3%) [f]
Ratio [r] of US [d] to FB mean diameters	0.980 (0.6%) [f,e,o]	0.988 (2.2%) [f,c]	1.060 (1.5%) [f,e]	1.082 (2.2%) [f,e]
Ratio [r] of SS [d] to EB mean diameters	0.984 (0.9%) [f,e,o]	0.990 (2.4%) [f,c]	1.043 (1.3%) [f,e,u,*]	1.064 (1.9%) [f,e]
Study of larger testes				
Area of FBs [a] (mm^2)	90.8 (6.6%)	43.4 (10.7%)	88.8 (5.6%)	41.9 (14.6%)
Ratio [r] of EB [a] to FB areas	0.888 (5.0%) [f,o]	0.905 (6.4%) [f,c]	1.037 (2.5%)	1.076 (8.2%) [f]
Ratio [r] of US [a] to EB areas	0.929 (3.9%) [f,e,o,l]	0.929 (5.8%) [f,e,c]	1.039 (3.6%) [f,*,l]	1.138 (5.5%) [f,e]
Ratio [r] of SS [a] to EB areas (i.e. *RAC*)	0.935 (4.7%) [f,e,o,l]	0.957 (6.5%) [f,c]	1.034 (4.2%) [f,*]	1.112 (3.4%) [f,e]
Mean diameter of FBs [d] (mm)	10.7 (2.7%)	8.8 (3.5%)	10.7 (3.6%)	8.0 (5.5%)
Ratio [r] of EB [d] to FB mean diameters	0.950 (0.7%) [f,o]	0.937 (2.3%) [f,c]	1.014 (2.2%) [f,]	0.994 (3.4%)
Ratio [r] of US [d] to FB mean diameters	0.966 (0.6%) [f,e,o,l]	0.977 (2.0%) [f,e,c]	1.025 (1.6%) [f,e,*,l]	1.063 (2.3%) [f,e]
Ratio [r] of SS [d] to EB mean diameters	0.968 (1.0%) [f,e,o,l]	0.972 (2.1%) [f,e,c]	1.011 (1.3%) [f,u,*,l]	1.053 (2.3%) [f,e]

Data: mean (%, coefficient of variation) calculated from each sub-group (n=6). **Design:** 12 smaller testes from 6 normal pubertal Sprague-Dawley rats and 12 larger testes from 6 normal mature Sprague-Dawley rats were used for study. From one testis (left or right alternately chosen) of each rat, 2 circular, central, adjacent tissue slices were cut perpendicular to the testicular long axis, with 1 slice (O-block) and one half (C-block) of the other slice being embedded in paraffin; 1 O-block and 1 C-block were obtained in the same way from the other testis of the rat and were embedded in methacrylate. Sections were cut along the central cut-surface of the embedded tissue blocks. **Abbreviations:** FB, the fresh tissue block face; EB, the embedded tissue block face left after the unstained section was cut; US, the unstained section cut from the embedded block; SS, the stained section, after staining and coverslipping of the unstained section; *RAC*, the ratio of (testicular section) area change. **Statistical tests results:** significantly different ($p < 0.05$) from FB [f], EB [e] or US [u] in comparison of the areas [a] (not ratios) or diameters [d] (not ratios) of FB, EB, US and SS (same column); $p < 0.05$ in comparison of the same ratios [r] between paraffin and methacrylate O-blocks [o] or C-blocks [c] (same line), between paraffin or methacrylate O-blocks and C-blocks * (same line), or between smaller and larger testes [l] (same column).

Slicing and embedding of tissue blocks

Two circular, adjacent slices (thickness 2-3 mm) were cut from the middle of each testis, perpendicular to the long testicular axis (with an approximate length of 13 mm and 20 mm for smaller and larger testes respectively). One slice (circular O-block of testicular tissue) and one half (semi-circular C-block of tissue) of the other slice (chosen randomly and cut along the circular center) were embedded in a mould separately, with care being taken to ensure that the central cut-surface of either tissue block was the cut-surface of the embedded block in sectioning with microtome. The O-block and C-block obtained from one testis (left or right alternately chosen) of one rat were embedded in paraffin and the 2 blocks obtained from the other testis of the rat were embedded in methacrylate (see Table 2 for such grouping of blocks), using the same embedding mediums and methods described above (Part "Sampling and embedding of tissue blocks").

Sectioning and staining of tissue sections

Sections were cut and stained in the same way as described above, with e.g. the same microtome and knives, the same block advance (paraffin 14 µm, methacrylate 25 µm), and the same mounting methods and staining protocols. Special attention paid or different treatment given included (i) a bit of the upper-right corner of the paraffin or methacrylate block was cut off before sectioning and the corner of the section cut from the block was placed at an upper-right position on the glass slide. Doing so was to better determine the cutting direction of sections (below). (ii) Only one section was obtained from each block. Effort was made to ensure that the section was intact, well cut and complete, and it was the first intact section that was obtained. (iii) Sections were stained with hematoxylin only (without periodic acid or Schiff's reagent), and, in particular, methacrylate sections were slightly stained with hematoxylin (15 minutes) so that the boundary of the tissue sections was clearer on the scan images (below). [The average actual section thickness, as measured in the same way as described above, of the 12 stained sections (6 from O-blocks and 6 from C-blocks for paraffin or methacrylate) was 14.5 µm (CV 7.2%) for paraffin and 20.0 µm (8.0%) for methacrylate.]

Scanning of tissue blocks and sections

The FB (fresh block face, central cut-surface of the testicular tissue block), EB (embedded block face left after the intact section was cut), US (unstained section, the intact section before staining) and SS (stained

section, the intact section after staining and coverslipping) were scanned (resolution 1200 ppi) with the same scanner (MRS-4800U2, Microtek, China) (see Fig. 3). Of note, in order to get a better scan image (clearer boundary) of EB, the block face was immersed in hematoxylin and water (depth 1-2 mm) before scanning for a while: 10 minutes in hematoxylin, 5 minutes in tap water and 1 minute in distilled water for paraffin blocks, and 1 minute in hematoxylin, 30 seconds in tap water and 30 seconds in distilled water for methacrylate blocks. (The EB was also scanned before the immersion procedure and an analysis showed that the procedure did not affect the size or shape of the block face.)

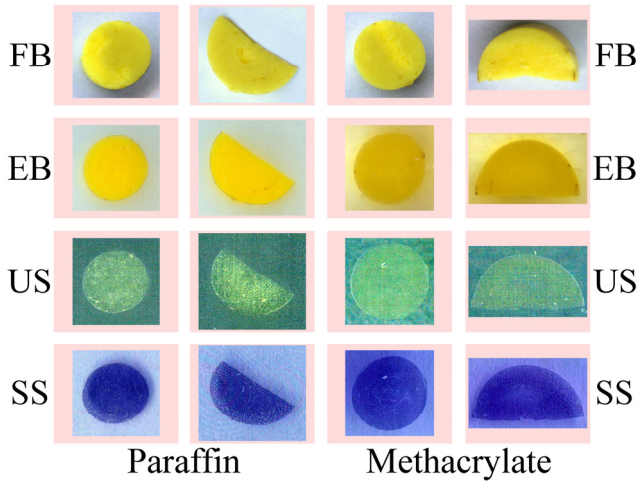

Fig. 3. *Scanned pictures of testicular tissue blocks (before and after embedding in paraffin or methacrylate) and the sections cut from the blocks. The 2 O-shaped and 2 C-shaped blocks were obtained from smaller testes (pubertal rats) and larger testes (mature rats), respectively. FB: a fresh block showing the testicular cut-surface; EB: the embedded block showing the cut-surface after the unstained section was cut; US: the unstained section on glass slide; SS: stained section, the US after staining with hematoxylin. Image processing of the scanned pictures with Photoshop: automatic contrast. The width and height of each small rectangular panel (pink background with a scanned picture placed on it): 12.5 and 10.1 mm.*

Measurement of scan images

Using an Adobe Photoshop 8.0.1 software as we previously described (Xiang *et al.*, 2018), the area of each scan image (Fig. 3), observed with a square grid (software generated) superimposed on it, was estimated by counting of test-points (intersections between the horizontal and vertical lines of the grid) as described above (Part "Estimation of total section area"). Different grids with different distances between the horizontal or vertical lines were used depending on the sizes of the images and an average of 40.3 (25-56) points were counted per image.

To confirm the results of areas (above) and to demonstrate possible section compression along the cutting direction, the diameters (length and width) of the images were also measured using the Photoshop (Xiang *et al.*, 2018). Before the measurements, (i) the image of each EB was rotated according to the horizontal edge (perpendicular to the cutting direction) of the supporting block so that the X-axis of the rotated EB is perpendicular to the cutting direction of sections. (ii) The image of each US was properly rotated according to the shapes of the US and the EB together with the edges of the embedding medium so that the X-axis of the rotated US is also perpendicular to the cutting direction. (iii) The image of each SS was properly rotated according to the SS's and the US's horizontal edges of the glass slide so that the X-axis of the rotated SS is perpendicular to the cutting direction as well. (iv) The image of each semi-circular FB (C-block) was properly rotated according to the cut-edges of the fresh and embedded C-blocks so that the X-axis of the rotated FB is approximately perpendicular to its cutting direction after embedding. (v) The image of each circular FB (O-block) was rotated to an independent, uniform random angle between 0 and 180 degrees (obtained with the Excel software) since it is difficult to determine which axis (direction) of the circular FB is perpendicular to its cutting direction after embedding.

Error analysis and statistical tests

To confirm accuracy of the above area estimation with the unbiased and efficient point counting method (Gundersen & Jensen, 1987), one stained section (SS) was arbitrarily chosen from each of the 8 sub-groups (Table 2) and its area was re-measured with the same method (above) for 2 times, independently and randomly. Before each re-measurements, the section image was cut from the original file and pasted into another image file, with arbitrary widths between the image edges and section edges, and was then rotated an independent uniform random angle between 0 and 180 degrees (obtained with the Excel). The CV (standard deviation divided by mean) of the three measurements (the original measurement plus these 2 additional independent re-measurements) was regarded as the *CE* of the original measurement (Yang, 2012), as a reference sampling error of the area estimation in the present study.

To detect statistically significant difference (defined as $p < .05$) between sub-groups, the one-way repeated measures analysis of variance was used for comparison of areas or diameters between FB, EB, US and

SS representing tissue change at different stages of tissue processing (Table 2 and Fig. 3). When significant difference was detected, the Student-Newman-Keuls method was further used for all pairwise multiple comparisons.

The paired *t* test was used to compare area-to-area or diameter-to-diameter ratios (not absolute results - areas or diameters) between O-blocks and C-blocks from the same testes to detect difference of degrees of tissue change between shapes/sizes of blocks/sections (Table 2). The paraffin O-blocks/C-blocks and the methacrylate O-blocks/C-blocks were from the same rats, and the paired t tests demonstrated that there were no significant differences ($p > .08$) between paraffin and methacrylate O-blocks' or C-blocks' FB areas (Table 2). So the paired t test was also used to compare results (ratios) between paraffin and methacrylate O-blocks or C-blocks to detect difference of tissue change degrees between embedding mediums (Table 2).

In addition, the *t* test was used to compare results (ratios) between smaller and larger testes to detect difference of tissue change degrees between testicular (section) sizes or textures (Table 2).

The paired t test was also used to compare the diameter ratio (ratio of the Y-axis diameter to the X-axis diameter) of SS and that of EB to determine if there was significant ($p < .05$) section compression along the cutting direction of sections (Table 3)

RESULTS

On the whole, the overall change (after processing) of the actual tissue volume of paraffin sections was a marked shrinkage of 21%-23% while that of methacrylate sections was a small expansion almost negligible (see *RVC(act)* in Table 1). The change of paraffin sections was largely contributed by tissue shrinkage at the process of embedding while that of methacrylate sections was first tissue swelling at the process of embedding and then section expansion (in area) at the process of section preparation (after sectioning), with the overall expansion being largely counteracted by a 15% of section compression in the actual thickness (Tables 1 & 2). Compared with area of the embedded block face, the final section area had a 4% decrease in paraffin but an 8% increase in methacrylate (Table 2).

Error Analysis

In the First Experiment, the average weight fractions of blocks sampled from the testes were 0.489 (CV 8.9%, paraffin) and 0.511 (8.5%, methacrylate) for the pubertal testes, 0.163 (8.5%) and 0.176 (9.6%) for the mature testes, and 0.064 (18.9%) and 0.065 (13.3%) for the adult testes, approximating the sampling fractions [SF(blo)] of 0.500, 0.167 and 0.063 for the three groups of testes (Table 1), respectively.

With respect to precision of the Cavalieri's estimates, the average *CE*s were 2.3% (CV 65.4%, paraffin) and 2.2% (54.2%, methacrylate), 1.2% (78.8%) and 0.8% (112.0%), and 2.1% (60.5%) and 1.9% (41.3%) for the pubertal, mature and adult testes, respectively.

As for the accuracy of the block advance (thickness set by the microtome), the average thickness of serial sections estimated by heights of the block and the number of serial sections was 24.94 μm (CV 2.4%) for the 18 blocks, close to the 25.00 μm set by the microtome.

In the Second Experiment, the *CE* of the area estimation for the 8 sections was 2.5% (CV 32.0%) on average.

Number of Non-intact Sections

In the First Experiment, among the sections cut at the two ends of the blocks there were always some sections which were non-intact, incomplete or with empty area around (or inside) the tissue section (Fig. 2). Artifact of apparent tissue detachment (Fig. 2) was not considered in this respect. Of the 25-35 serial sections sampled and stained per sub-group (Table 1), the number of non-intact sections accounted for 23%-34% (paraffin) and 24%-29% (methacrylate), see Fig. 2.

Table 3: Results indicating section compression along the cutting direction (Second Experiment)

	Paraffin embedding		**Methacrylate embedding**	
	O-blocks (n=6)	**C-blocks (n=6)**	**O-blocks (n=6)**	**C-blocks (n=6)**
Study of smaller testes				
Diameter ratio (Y/X) of SS	0.896 (5.6%) [e]	0.786 (23.5%) [e]	0.974 (3.6%)	0.577 (6.8%)
Ratio of SS's to EB's diameter ratios	0.913 (2.8%)	0.911 (2.4%)	0.988 (3.8%)	0.963 (4.0%)
Study of larger testes				
Diameter ratio (Y/X) of SS	0.926 (4.4%) [e]	0.973 (39.7%) [e]	0.999 (4.6%)	0.642 (5.6%)
Ratio of SS's to EB's diameter ratios	0.916 (1.1%)	0.913 (3.9%)	0.983 (3.3%)	0.991 (1.8%)

Data shown as mean (%, coefficient of variation). SS, the stained section; EB, the embedded block face; diameter ratio (Y/X), ratio of the Y-axis (cutting direction) diameter to the X-axis diameter of the SS or EB. [e]The diameter ratio (Y/X) of SS significantly different ($p < 0.05$) from that of EB (this ratio not shown in the Table). See the footnote of Table 2 for the study design and other abbreviations.

In the 8 sub-groups of the Second Experiment, the average number of testicular sections (counting from the first section cutting the testicular tissue) discarded before the first intact section was obtained for study was 15-20 (O-blocks and C-blocks) and 25-36 for paraffin sections from smaller and larger testes and 36-37 and 60-87 for methacrylate sections from smaller and larger testes, respectively.

Comparison of Section Sizes between Unstained and Stained Sections

In general, there were not marked differences between the areas or mean diameters of stained sections (SSs) and those of the unstained sections (USs), as can be seen in the ratios of SS or US sizes to those of EB (embedded block face) sizes (Table 2). Significant difference was detected only in the diameters of the 2 subgroups of methacrylate O-blocks: the mean diameter ratios of SS to EB were 1.4%-1.6% smaller than those of US to EB (Table 2).

Section Diameters and Compression along the Cutting Direction

As can be seen in the size ratios between EB and FB, US and EB, and SS and EB (Table 2), the mean diameter changes were generally consistent with the area changes. An exception was found only in the sub-group of paraffin O-blocks (smaller testes) where the diameters of USs or SS were 1.6%-2.0% smaller (with statistical significance) than those of EBs whereas there were not significant differences in the areas between US (or SS) and EB (Table 2).

In the 4 sub-groups of paraffin sections, the Y-axis (cutting directions of sections) diameter to X-axis diameter ratios of SSs were 8.4%-8.9% smaller (with statistical significance) than those of EBs, indicating a 8.4%-8.9% linear compression of sections along the cutting direction (Table 3). In methacrylate sections, however, significant section compression along the cutting direction was not detected (Table 3).

Paraffin Sections: Tissue Volume and Section Area Changes

The actual ratio of volume change [*RVC(act)*] of testicular tissue after paraffin processing was 0.77-0.79 (a change from beginning to end of processing, Table 1), which indicated a 21%-23% tissue shrinkage (in volume) after processing, without significant difference between the 3 groups of testes (one-way analysis of variance). Considering an increase of ~4% in the actual section thickness (compared with block advance), the overall volume shrinkage not considering the thickness change would be ~25%, as indicated by the block advance based ratio of volume change [*RVC(ba)*] in Table 1. The ratio of section area change (*RAC*) was around 0.96 (0.94-1.00) overall (a change after cutting) in the 4 paraffin sub-groups (Table 2), indicating a 4% decrease of the section area compared with the area of the embedded block face. This areal decrease, which we assume herein was a result of uniform volume (virtual) shrinkage, would induce a volume decrease of ~6% [$=1-(0.96^{3/2})$], which suggests that the change after cutting (areal shrinkage) and the change after embedding (volume shrinkage from beginning to embedding) accounted for approximately 1/5 and 4/5 of the 25% volume shrinkage, respectively, if we assume results in Tables 1 and 2 are mutually explainable.

The area ratio of the embedded block (EB) to the fresh block (FB) was around 0.90 (0.89-0.93) for the 4 paraffin sub-groups (Table 2), indicating a 10% areal shrinkage after embedding (from fresh block to embedding). This areal shrinkage would induce a volume decrease of ~15% [$=1-(0.90^{3/2})$], slightly smaller than the 20% volume shrinkage from beginning to embedding (4/5 of the 25% overall volume shrinkage above). This might suggest that the fresh block obtained might have ~5% of shrinkage once cut from the testis.

As a result of the section area change, the virtual ratio of volume change [*RVC(vir)*] was approximately 2% smaller than the block advance based ratio of volume change [*RVC(ba)*], see Table 1.

Methacrylate Sections: Tissue Volume and Section Area Changes

The *RVC(act)* of testicular tissue after methacrylate processing was 1.01-1.03 (Table 1), which indicated a 1.6% (on average) tissue expansion (an almost negligible tissue change) in volume after processing, without significant difference between the 3 groups of testes (one-way analysis of variance). If not considering a marked decrease of ~15% in the actual section thickness compared with block advance, the volume change would be a marked expansion of 20% (16%-23%) as indicated by the *RVC(ba)* in Table 1. The *RAC* of methacrylate sections was around 1.08 (1.03-1.11) overall (a change after cutting) in the 4 sub-groups (Table 2), indicating an 8% increase of the section area compared with the area of the embedded block face. This areal increase, which we assume was a result of uniform volume (virtual) expansion, would induce a volume increase of ~12% [$=(1.08^{3/2})-1$), which suggests that the change after cutting (areal expansion) and the change after embedding (volume swelling from beginning to embedding) accounted for approximately 2/3 and 1/3 of the 20% volume expansion, respectively.

The area ratio of EB to FB was around 1.06 (1.04-1.08) for the 4 methacrylate sub-groups (Table 2), indicating a 6% areal expansion after embedding (from fresh block to embedding). This areal expansion would induce a volume increase of ~9% [=($1.06^{3/2}$)-1], slightly larger than the ~7% volume expansion from beginning to embedding (1/3 of the 20% overall volume expansion above). This might indicate a slight shrinkage of the fresh block once cut from the testis.

Note, if the section area increase (8%) was associated with a proportional decrease of the section thickness (i.e. assuming that the area increase was not associated with the volume change), the thickness decrease would be about ~7.5% [≈1-(1/1.08)]. So the decrease of 15% in the actual section thickness (above) might be equally contributed by section flattening due to areal expansion of the elastic plastic section and by section compression (in thickness) due to other factors such as heating on hot plate.

As a result of the section area change, the *RVC(vir)* was approximately 5% larger than the *RVC(ba)*, see Table 1.

Factors Affecting the Section Area Change

As can be seen in the area ratios of EB to FB, or SS to EB (Table 2), (i) paraffin and methacrylate tissue changes during processing were markedly different, with paraffin embedded tissue showing essentially shrinkage while the methacrylate embedded tissue showing expansion. (ii) There were not significant differences between O-blocks and C-blocks (paraffin or methacrylate) except that the methacrylate *RAC* of C-blocks was significantly larger than that of O-blocks, suggesting that methacrylate sections or blocks without a complete capsule might have a larger expansion. (iii) There were not significant differences between smaller and larger testes or sections (O-blocks or C-blocks) except that the larger testis's *RAC* of paraffin O-blocks was significantly smaller than the smaller testis's *RAC*, suggesting that larger paraffin O-blocks from larger testes might have a larger shrinkage.

DISCUSSION

Theoretical Consideration

Stereological (quantitative) estimation is performed on final tissue sections that have undergone a series of tissue processing from the original organ. For global estimates of structures in an organ such as total number, length or surface area, the change of the tissue volume or section area after processing may need to be estimated to correct the results obtained on sections so as to reflect the true results in the original organ (Part "Stereological Background"). The present study proposed that the correction be based on the stereological principle used for the estimation and the actual or virtual ratio of volume change be estimated for the correction. The actual and virtual ratios, or the virtual thickness and volumes (Equation 8), are new concepts proposed in the present study, but not very difficult to understand (see Part "Stereological Background").

Specifically, for total number (of particles) estimation with disectors (based on 3D measurements), the actual tissue volume change (or the actual ratio of volume change) should be estimated for correction (Equation 17); for total number estimation, or total length or surface area estimation, based on 2D measurements, the virtual volume change (or the virtual ratio of volume change) should be estimated for correction (see Equation 18 and the last paragraph in Part "Stereological Background"). Of note, both tissue volume change (the block advance based ratio of volume change, to be precise) and section area change (or the ratio of section area change) are needed for estimation of the virtual volume change (see Equations 10, 14 & 16).

Methodological Consideration

Using paraffin and methacrylate embedded testicular sections, the present study estimated the actual and virtual ratios of the tissue volume change. Based on experience from previous researches (see the second last paragraph in Part "Study of Tissue Volume and Section Area Changes after Processing"), the present study was designed not only comprehensively but also in an unbiased way. The unbiasedness consists mainly (i) in the use of the stereological Cavalieri's principle combined with the fractionator (for volume estimation), in the combined use of the actual section thickness (measured directly with a microcator), and in the careful design that enabled estimation of the overall change from the original organ in the First Experiment, and (ii) in the same way of clearly observing and measuring section images (Fig. 3), in the area estimation with point counting, and in the careful design that included all major steps of tissue processing in the Second Experiment.

"There is no 'smart' unbiased way to obtain information about tissue deformation during tissue fixation and processing" (Nyengaard, 1999). Complete serial sections of embedded blocks (Fig. 2) are often needed to estimate the volume of organ after processing, which is essential for tissue volume change study. Use of serial sections means the need for more experience in histological technique and also more time and effort. In addition, serial sections are not all perfect, with at least many intact sections (see Fig. 2 and Part "Number of Non-

intact Sections"). Sadeghinezhad & Nyengaard (2019) used single sections to represent tissue blocks, a simple method of avoiding the use of serial sections, but apparently a biased method. We may also obtain the areal change of tissue before and after processing to estimate the tissue volume change (Nyengaard, 1999). This is another simple method often used to avoid serial sections, but apparently a biased method as well. Nevertheless, the average area ratio of the stained section (SS) to the fresh block face (FB) for paraffin in the Second Experiment (Table 2) was 0.87 (0.83-0.89), therefore the volume shrinkage estimated with this areal method would be ~19% [=1-$(0.87)^{3/2}$], which happened to be close to the actual volume shrinkage of 21%-23% we estimated in the First Experiment (Table 1). This suggests that the tissue change in paraffin was relatively consistent at different steps of processing, especially in embedding where the major change occurred. So this areal method may be tentatively used for approximate estimation of tissue shrinkage of paraffin sections. Apparently this method is not suitable for methacrylate sections where the actual volume change was a marked tissue expansion during processing (Table 2) neutralized by a marked tissue shrinkage due to reduction in the actual section thickness (Table 1).

Linear change (change of length or width) of tissue blocks or sections was also used previously to reflect tissue change (Weibel, 1979; Gerrits *et al.*, 1992). This is more unadvisable as it is difficult to measure them (at all steps of processing), its results are affected by non-uniform change of the tissue, and its error, if any, will be augmented when converted to volume change. Take the diameter measurement in the present study as an example, we were confident to determine the cutting direction (Y-axis) of the embedded block, but this axis might be different or deformed for the fresh block, unstained section or stained section and could not be determined with full confidence. Measurement or comparison of diameters was only an approximation, for confirmation of the area results.

Measurement of actual section thickness is important for estimation of the actual ratio of volume change. There are no other measurement methods that are simpler or better than the one with a microcator (Part "Measurement of actual section thickness"). Our experience for accurate measurement is that (i) the top or bottom surface of the tissue section is determined while the serial optical focal planes are moving up or down slowly and smoothly (through the thick section) with turning of the microscope adjustment knob, and (ii) the tissue structure which begins to appear or disappear on the top or bottom surface includes not only nuclei but also their surrounding tissue, with granules of nuclear chromatin being the key reference structure no matter whether they are darkly or lightly stained. In the present study, the mean actual thickness of methacrylate sections (with the same block advance) was 6% smaller in the Second Experiment (Part "Sectioning and staining of tissue sections") than that in the First Experiment (Table 1), probably because of the markedly lighter staining in the Second Experiment. Interestingly, we additionally tried and measured a few methacrylate sections (with the same block advance) which we used in other studies years ago, and obtained a result of the actual section thickness comparable with the present study.

Previously tissue blocks cut from organs were often used to estimate tissue volume change, i.e. the volumes of the blocks before and after processing were taken as the V(pre) and V(post) in Equation 6 (e.g. Zhengwei *et al.*, 1997). In this case, attention should be paid to accuracy of the weight and density measurements (to estimate the volume before processing) for small blocks or blocks without a complete capsule. In particular, the present study demonstrated with both paraffin and methacrylate sections that the volume of fresh tissue blocks cut from a testis was a few percents smaller than the volume of the “original” blocks within the original testis (Parts "Paraffin Sections: Tissue Volume and Section Area Changes" and "Methacrylate Sections: Tissue Volume and Section Area Changes"). This may be explained by the fact that the testis is of considerable intratesticular pressure (Ma *et al.*, 2016), thus once the testis is cut into blocks, the blocks (or the “testis” reconstructed from these blocks) may shrink to some degree.

For estimation of tissue volume change in the present study, the volume of testis after processing was compared with the volume before processing. But the so-called “volume before processing” herein was actually a volume at the stage of storage in 70% ethanol after fixation (Part "First Experiment: Study of Tissue Volume Change"), not the volume of the *in vivo* testis or the original fresh testis before any processing. But this is out of practical consideration: at the stage of storage in 70% ethanol researchers can have more time and be more focused on the works of organ dissection and measurements. Moreover, we previously demonstrated that volume of the fresh testis (just removed from rats) did not change markedly after immediate fixation in Bouin’s fluid and dehydration in 70% ethanol (Zhao *et al.*, 2006).

Tissue Changes

We previously demonstrated that paraffin embedded testicular sections (block advance 5 or 10 μm) had an areal shrinkage (SS compared with EB) of

5.5%−8.6% and a linear section compression (along the cutting direction) of 5.9%−8.9% (Xiang *et al.*, 2018). In the present study (block advance 14 μm), the shrinkage and compression results were ~0-6.5% and 8.4%-8.9%, respectively. This might suggest that thinner paraffin sections had a slightly larger areal shrinkage. In contrast, the present study demonstrated marked areal expansions with methacrylate sections (Table 2): expansion by 3%-11% (SS compared with EB) or 7%-19% (SS compared with FB). Marked areal expansion of methacrylate sections was previously noted during section stretching and mounting, but with final section size being comparable with that of the original block face (Gerrits *et al.*, 1992).

It was thought that paraffin sections might have volume shrinkages up to 50% (Dorph-Petersen *et al.*, 2001; Yang, 2012). Such large shrinkage might be possible with some paraffin or knives used previously (e.g. Yang & Cui, 1989), but the present study using the Leica paraffin and knives together with an unbiased design demonstrated a much smaller shrinkage: 21%-23% (as indicated by RVC(act) in Table 1). With paraffin sections of renal tissues, a volume shrinkage of 17% was found by Akbari, Goodarzi & Tavafi (2017) using the areal method (Nyengaard, 1999) and a volume shrinkage of 25% was shown by Sadeghinezhad & Nyengaard (2019) using single sections.

It was concluded from a limited study that tissue (actual volume) shrinkage of methacrylate sections was negligible (Zhengwei *et al.*, 1997; Yang, 2012). And indeed, as demonstrated in the present study of methacrylate sections, the actual tissue volume change was almost negligible. But the overall change was a volume expansion (indicated by a section area expansion of 7%-19%, see above) counteracted by a volume shrinkage (indicated by a section thickness compression of 15%, Table 1), and 2/3 of the area expansion was contributed by expansion after cutting (Part "Methacrylate Sections: Tissue Volume and Section Area Changes"). So it seems that the tissue change of methacrylate sections is more "manipulable", thus more attention should be paid to "standard" cutting, mounting and staining of methacrylate sections in practice

Implications

Considerable volume changes of paraffin and methacrylate embedded testicular sections shown in the present study, especially the actual volume changes of paraffin sections and the virtual volume changes of both paraffin and methacrylate sections (Table 1), indicate the importance of study of tissue changes and correction of stereological results in practical stereological studies.

Some of previous studies by ZWY and co-authors (e.g. Zhengwei *et al.*, 1997; Zhang *et al.*, 2002) estimated (total) nuclear numbers per testis by "the numerical density × the volume of fresh testis" where the density was obtained with the optical disector using methacrylate sections. Assuming the actual volume change of the testicular tissues after processing in the studies to be similar to the change shown in the present study, the number estimation could be tentatively corrected by multiplying the *RVC(act)*: ~1.016 (Table 1), almost negligible. For estimation of the total area of alveolar surface per lung with paraffin sections by "the surface density × the volume of fresh lung" (Yang *et al.*, 2002), the result could be tentatively corrected by multiplying "$RVC(vir)^{1/3}$" where *RVC(vir)* is ~0.74 (Table 1). For estimation of the total length of renal tubules per kidney with methacrylate sections by "the length density × the volume of fresh kidney" (Wang *et al.*, 2021), the result could be tentatively corrected by multiplying "$RVC(vir)^{2/3}$" where *RVC(vir)* is ~1.26 (Table 1). That is, the total surface area and length estimates could be multiplied by 0.90 and 1.17 respectively for correction.

Not many applied researches with stereological methods addressed the potential effects of tissue volume change after processing on the results, probably because of ignorance, tolerance or inability (e.g. without a stereology system equipped with a microcator to measure actual section thickness), because of difficulty in understanding or design, or because of need for much more time, effort or cost. Our suggestions are: (i) For comparative studies, use of materials and methods, even including the size and shape of tissue blocks, should be standardized for all groups. (ii) Methods used, even including the section heating and staining, should be clearly stated, leaving the tissue change issue open for discussion. (iii) Correction coefficients (ratios of volume change) may be tentatively cited for correction if the tissue processing procedures are similar. Tissue volume change after histological processing is essentially mechanical or physical, perhaps without marked difference between studies if the materials and methods used (such as organ or tissue blocks, embedding medium, cutting instruments and section thickness & staining) are comparable. (iv) If a series of (stereological) studies are being conducted by a lab or group, at least one study may be considered for estimation of tissue volume change. (v) For estimation of total particle number, a method of disector combined with fractionator may be used, where the reference volume need not be known, thus a correction of tissue volume change is not necessary (Gundersen *et al.*, 1988b; Nyengaard, 1999; Dorph-Petersen *et al.*, 2001; Yang, 2012). Such fractionator method requires the strictest sampling design in stereology and such design usually

involves serial sections. (vi) Volume is a basic, universal property of any structures and the total volume estimation does not need a correction for tissue volume change (see the last paragraph in Part "Stereological Background"). So when study objective could be realized with the parameter of total volume, it is advisable to focus on volume estimation. And when many stereological parameters are obtained (e.g. Ma *et al.*, 2016; Guo *et al.*, 2019; Wang *et al.*, 2021) we may pay more attention to the parameter of total volume.

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