Alterations of mouse lung tissue dimensions during processing for morphometry: A comparison of methods

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Preservation of original tissue dimensions is an essential prerequisite for morphometric studies. Shrinkage occurring during tissue processing for histology may severely influence the appearance of structures seen under the microscope and stereological calculations. Therefore, shrinkage has to be avoided so that estimates obtained by application of unbiased stereology are indeed unbiased. The present study investigates the alterations of tissue dimensions of mouse lung samples during processing for histology. Different fixatives as well as embedding protocols are considered. Mouse lungs were fixed by instillation of either 4% formalin or a mixture of 1.5% glutaraldehyde/1.5% formaldehyde. Tissue blocks were sampled according to principles of stereology for embedding in paraffin, glycol methacrylate without treatment with osmium tetroxide and uranyl acetate, and glycol methacrylate including treatment with osmium tetroxide and uranyl acetate before dehydration. Shrinkage was investigated by stereological measurements of dimensional changes of tissue cut faces. Results show a shrinkage of the cut face areas of roughly 40% per lung during paraffin embedding, 30% during “simple” glycol methacrylate embedding, and <5% during osmium tetroxide/uranyl acetate/glycol methacrylate embedding. Furthermore, the superiority of the glutaraldehyde-containing fixative regarding shrinkage is demonstrated. In conclusion, the use of a glutaraldehyde-containing fixative and embedding in glycol methacrylate with previous treatment of the samples with osmium tetroxide and uranyl acetate before dehydration is recommended for stereological studies of the mouse lung.

Zones of the mouse lung; stereology; shrinkage; embedding; fixation

SCIENTIFIC PROJECTS USING histological methods aim either at qualitative or quantitative investigations of the organ of interest. In the lung, for example, qualitative studies may deal with the question whether certain morphological alterations compared with “normal” lung morphology are existent or not (e.g., inflammatory infiltration or alveolar remodeling). Quantitative investigations may attempt to measure the extent of these morphological alterations (e.g., reduction of alveolar surface area in pulmonary emphysema) (cf. Ref. 42). In both settings the aim is to draw conclusions about the in vivo state of lung architecture (cf. Ref. 56). However, investigation of histological sections has to deal with various problems (58): in contrast to the three-dimensional (3D) organ, histological sections are almost two-dimensional (2D), and their investigators have to live with the loss of most of the 3D information (42, 45). In quantitative studies the application of design-based stereology (for review, see Refs. 8, 20, 21, 23, 24, 29, 35, 38, 42, 45, 53, 54, 58) can handle this problem and allows the scientist to transfer the information gained from almost 2D sections to the third dimension.

Besides the loss of the third dimension, there is another critical issue that has to be taken into consideration. Although one wants to investigate the in vivo state of the organ, investigation of a microscopic section in general means investigation of an artificial product (38, 42, 55, 56). What happens to the organ before stereological measurements? In a first step, the organ has to be fixed and removed from the animal. Because in most cases one cannot investigate the entire organ, representative samples for further evaluation have to be taken (e.g., Refs. 12, 23, 29, 30, 45). These samples have to be embedded in an embedding medium (e.g., paraffin or plastic). From the blocks obtained this way, sections have to be taken, mounted on glass slides, and stained (cf. Ref. 10).

The initial fixation and embedding are crucial steps during tissue processing; the initial fixation is the basis for preservation of structural integrity before onset of autolysis of the organ while embedding facilitates obtaining histological sections and, furthermore, allows easy storage of the samples for a long time without further need of fixing agents (cf., e.g., Refs. 1, 18, 59). During embedding, however, the samples come in contact with several different chemicals (e.g., alcohols for dehydration) that may alter the tissue.

Shrinkage is a critical phenomenon that can happen to the tissue (10, 38, 42, 58, 60). Besides having influence on the appearance of structures under the microscope (qualitative level), shrinkage can severely influence quantitative measurements, in particular measurements regarding object size (60). A widely used stereological approach is the so-called “density times reference volume design” (38), meaning that in a first step a certain parameter is estimated microscopically as a density (e.g., alveolar surface area/unit volume of parenchyma), and in a second step the density is multiplied by the volume of the corresponding reference space to end up with absolute values (for a more detailed description see Ref. 55). This procedure requires knowledge of the total organ volume, which can be estimated by either Scherle’s method (44, 45) or the Cavalieri method (23, 32, 45), for example. In general, these estimations are based on the entire organ (Scherle’s method) or on tissue slabs created during sampling (Cavalieri method) and therefore on tissue conditions at the beginning of tissue processing (in particular before dehydration and embedding), so that subsequent tissue shrinkage occurring during embedding may severely influence other estimates (see also 10, 36, 42). Shrinkage therefore has to be avoided (10, 60).
The aim of the present study was to investigate the influence of the initial fixation and different embedding protocols on tissue dimensions of mouse lungs during processing. For “simple” light microscopy, widely used protocols include formalin fixation and embedding in paraffin, so they are included in this study.

In terms of shrinkage, plastic embedding [e.g., in glycol methacrylate (GMA)] is considered to be superior to paraffin embedding for stereology (24, 58) and therefore recommended for quantitative morphometric analyses (24, 38, 60). Support for this opinion can be found in the literature (9, 33, 61). Therefore, there are good arguments to analyze GMA as an alternative to paraffin embedding in the present study (for detailed review of plastic embedding see Ref. 17). As “end point” of this study, the time point directly before the final paraffin or plastic blocks were poured in the molds was chosen.

As an alternative to formalin fixation, we chose a mixture of glutaraldehyde (GA) and formaldehyde (freshly prepared from depolymerized paraformaldehyde, therefore from now on abbreviated PFA) (see Refs. 18, 26). GA (which is in contrast to formaldehyde chemically a diadehyde) is widely used as the primary fixative for conventional transmission electron microscopy because of its good ultrastructural preservation of tissues. Both formaldehyde and GA stabilize tissues by cross-linking proteins via amino groups, but the cross-linking by GA is deemed more stable than that by formaldehyde. However, because of its faster penetration in tissues and therefore its initial fixation, formaldehyde is often added to the primary fixative. Because GA is thought to be superior to formaldehyde fixation in terms of tissue fixation (58), one could assume that GA-fixed lungs might retain the volume at which they were initially fixed better than formalin-fixed lungs (see also Ref. 62).

For electron microscopy, contrast and fixation of tissues can be enhanced by postfixing the samples with osmium tetroxide (OsO4) and staining them with uranyl acetate (UA) (18). Both agents react with lipids, and a sequential use of both results in a cross-linking effect on membranes and cytoplasm become well preserved by cross-linkage of the different structures involved (18). Data from Nielson and Griffith (37) suggest that OsO4 has a direct cross-linking effect on proteins.

UA has a staining effect on tissue structures, in particular membranes. Besides this staining effect, it also has a fixing effect on membranes (18, 48) because of phospholipid stabilization (18), as well as on proteins and nucleic acids (18), and can therefore further enhance the preservation of ultrastructure.

Because pulmonary surfactant, intra-alveolar or intracellular in the form of lamellar bodies of alveolar epithelial type II cells, contains a lot of lipids, especially phospholipids, post-fixation and staining of samples for light microscopy with OsO4 and UA appears advantageous to sufficiently map those structures essential for normal lung function morphologically to histological sections (38). Indeed, the ability of UA to preserve the ultrastructure of lamellar bodies has been demonstrated previously (13), and the enhanced preservation of the alveolar lining layer by OsO4 and UA (55) is desirable. Furthermore, experience (38) and data from the literature (5) suggest that osmication and treatment with UA before dehydration and embedding may counteract shrinkage of the lung considerably. Therefore, some samples of the plastic group were treated with OsO4 and UA before dehydration.

**MATERIALS AND METHODS**

**Fixation and Organ Preparation**

The study was approved by the local ethics committee (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit). Lungs from ten 18-wk-old male C57BL/6NCrl wild-type mice (Charles River, Sulzfeld, Germany) were fixed by airway instillation (e.g., Refs. 12, 55, 62), either by 4% formalin in 0.15 M HEPES buffer (formalin stock solution: formaldehyde solution min. 37%, free from acid; Merck, Darmstadt, Germany) or by a mixture of 1.5% GA and 1.5% PFA in 0.15 M HEPES buffer (5 lungs/group) (compare Refs. 36, 50).

After an intraperitoneal injection of ketamin/xylazin/heparin and clinical signs of death, the trachea was carefully exposed, and a 22-gauge cannula was inserted via a small incision. The trachea was then ligated around the cannula. Afterward, the peritoneal cavity was opened, and an incision was made in the diaphragm on either side to induce a bilateral pneumothorax. Before the fixation was started, each pleural cavity was flushed with 2 ml of 0.9% NaCl solution to prevent pleural adhesion by the fixative or to dissolve existing adhesion. The pneumothoraces were extended to enable efflux of the NaCl solution. Fixation was then started by instillation of fixative via the cannula connected to a fixative reservoir with an initial fluid level of about 25 cm above the table. After 10–24 s when the fluid had finished, the cannula was removed from the trachea while the trachea was ligated simultaneously. After the retroperitoneal vessels were pinched off, the thoracic organs were carefully removed en bloc, and the hilum pulmonis was carefully dissected in situ so that both vessels and airways could be ligated close to the lung. Vessels and airways were cut through shortly above the ligature. Up to the first volume measurement by Scherle’s method (44) the left and right lung were put in the particular fixative.

**Volume Determination and Sampling of Tissue Blocks for Different Embedding Media**

The total volume of the fixed lungs was estimated by Scherle’s method (44, 45). Before each measurement, the surface of the organs was gently swabbed with small cotton sticks to absorb adherent liquid. Scherle’s method was applied using pure water. At each time point, three measurements were taken, which were used to calculate an arithmetic mean. The lungs were sampled according to systematic uniform random (SUR) sampling (e.g., Refs. 12, 23, 29, 30, 45).

The lungs were embedded in 4% agar that was poured directly in a tissue slicer (e.g., Ref. 32). Once the agar had hardened, two knives were pushed in the agar block on either side of the lungs, and the length of the block in between was measured with a ruler, rounded to the full millimeter. By means of the tissue slicer, the agar block was then cut into slabs of roughly the same thickness. (The average thickness is the previously measured block length divided by the number of slabs.) Subsequently, the slabs were allocated so that always the same cut face pointed upward. The agar slabs were separated between the right and left lung, and a photo of each slab was taken (Nikon D80 with macro objective Nikon AF-S Micro Nikkor 105 mm 1:2.8 G ED) to enable an estimation of the total lung volume based on the Cavalieri method (32, 45, 62).

The surrounding agar was removed, and all tissue pieces were arranged in a smooth arrangement (small-big-small) (19). Based on this arrangement, every third piece of tissue was assigned to one of the three embedding protocols (i.e., paraffin and GMA with or without OsO4 and UA) according to SUR sampling. The order of the first triplet was determined by throwing a die (Fig. 1).
On day 3 the samples were transferred to pure hardener I. After at least 48 h of infiltration with hardener I, the plastic blocks were poured out in the molds on day 5 (hardener I + hardener II). The samples that were chosen to be followed during the embedding procedure were embedded separately.

Paraffin embedding. Paraffin embedding started on day 3 of the embedding procedure. The samples were transferred to 70% isopropanol for infiltration overnight. The next day (day 4) the samples were put in an automated embedding machine (Shandon Citadel 1000) where they were treated with isopropanol (70%, 90%, 100%), methyl benzoate, and Roti-Histol (Karl Roth, Karlsruhe, Germany) one after another before they were finally infiltrated by liquid paraffin. On day 5, the paraffin blocks were pour out. Similar to plastic embedding, the samples chosen to be followed during the embedding procedure were embedded separately.

Quantification of Shrinkage

To quantify the shrinkage during the different embedding procedures, the cut face of the previously chosen tissue blocks was estimated by point counting (8, 21–23, 29, 32, 53, 61). By means of image manipulation software GIMP 2.8 (www.gimp.org) a square including the tissue block and the ruler was cut out of the photos, which were taken during sampling and embedding. The size of the squares (side length in pixels) was kept constant within one lung and was determined by the size of the tissue blocks on the photos.

By means of the freely available stereology tool STEPanizer (49) (www.stepanizer.com) a regular point grid was superimposed on the processed photos, and the cut face area $A$ of each slab was estimated by counting all points $P$ hitting the tissue cut face and multiplying these points by the associated area per point $a(p)$:

$$A = \sum P \cdot a(p)$$ (1)

Area estimation by point counting is illustrated in Fig. 3. The estimated areas $A_i$ of each slab of one lung and the particular embedding protocol at certain time points were added up to the total area $A_{\text{total}}$ per lung, which was investigated during the particular embedding protocol (cf. Ref. 61):

$$A_{\text{total}} = \sum A_i$$ (2)

These total areas ($A_{\text{total},x}$) per lung, embedding protocol, and time point were related to the particular total cut face area at the time point “before embedding” ($A_{\text{total},\text{be}}$) so that relative cut face areas ($A_{rel,x}$) were obtained with a cut face area of 100% at the time point before embedding:

$$A_{\text{rel},x} = \frac{A_{\text{total},x}}{A_{\text{total},\text{be}}} \cdot 100$$ (3)

The relative cut face areas were used to calculate a mean per lung for each time point during the different protocols (for review of shrinkage quantification cf. also Refs. 10, 14, 24, 25, 36, 40, 60). Table 1 shows how many slabs per fixation and embedding protocol were investigated, whereas Table 2 shows how many points per lung on average were counted.

Statistical Analysis

A two-way ANOVA for repeated measurements using IBM SPSS Statistics 20 was employed to carry out statistical analyses of the different shrinkage results at the end point of this study. A $P$ value $< 0.05$ was considered significant.

RESULTS

Figure 4 demonstrates the shrinkage during the different embedding protocols qualitatively. The top row shows three
similar tissue blocks that in each case underwent one of the three different embedding protocols (i.e., paraffin or GMA without/with OsO4 and UA). The top row shows the tissue blocks at the time point “before embedding” while the bottom row shows the same tissue blocks before the final step of the embedding protocol (i.e., pouring out the paraffin or plastic blocks in the molds). Considering the paraffin and the plastic block without OsO4 and UA, there is considerable shrinkage observable, and the paraffin block has obviously shrunken most. In contrast, the plastic block that had been treated with OsO4 and UA seems to have shrunken hardly at all.

Figures 5, 6, and 7 show the shrinkage occurring during the different embedding protocols quantitatively and also distinguish between the two different fixatives used. The data are plotted as relative cut face area per lung [total cut face area of all slabs of one lung and the particular embedding medium, which have been quantified, related to the particular total cut face area at the time point before embedding (compare above)]. The group that underwent paraffin embedding showed a shrinkage of roughly 40% at the time point “before pouring out” (41.0% in the formalin subgroup and 37.2% in the GA/PFA subgroup, mean: 39.1%). Compared with this, the shrinkage in the GMA group without OsO4 and UA was less but still added up to roughly 30% (31.1% in the formalin subgroup and 27.5% in the GA/PFA subgroup, mean 29.3%). In contrast to paraffin embedding and simple GMA embedding, in the GMA group in which the samples had been treated with OsO4 and UA before dehydration, shrinkage was reduced to roughly 3% (3.1% in the formalin subgroup and 2.5% in the GA/PFA subgroup, mean 2.8%), which is a reduction by a factor of about 10 compared with simple GMA embedding. Comparing the different fixatives, the shrinkage in the GA/PFA-fixed subgroups was slightly less than the shrinkage in the formalin-fixed subgroups at the end point of the embedding procedures (before pouring out). Statistical analyses showed that the differences at the end point (before pouring out) between the different embedding protocols on the one hand (P < 0.01) and the different fixatives on the other hand (P < 0.05) were significant.
been estimated before the shrinkage occurred, if shrinkage is not corrected (36, 42). The estimated reference volume (e.g., by Scherle’s or the Cavalieri method) then no longer corresponds to the reference volume of the structures seen under the microscope (cf. Refs. 10, 24).

The aim of the present study was to compare the influence of different fixatives and embedding protocols on tissue dimensions during processing for histology. Therefore, we measured the cut face of differently fixed tissue blocks at different time points during different protocols of tissue processing. We found a mean area shrinkage of 39.1% for paraffin embedding, 29.3% for GMA embedding without osmication and UA, and only 2.8% for GMA embedding where the samples had been treated with OsO4 and UA before dehydration.

The differences are striking, but there are some aspects that have to be considered carefully: the measurements only reflect the shrinkage in two dimensions. Under the assumption that shrinkage along all three axes in space is equally high, the volume shrinkage $S_{\text{vol}}$ can be calculated, according to

$$S_{\text{vol}} = 1 - A_{\text{rel}}^{\frac{3}{2}}$$  \hspace{1cm} (4)

(see also Refs. 4, 24). We then find a volume shrinkage during paraffin embedding of 52.5% and a shrinkage for simple GMA embedding of 40.6%. In contrast, the volume shrinkage in the OsO4/UA/GMA group remains on a very low level of 4.2%, more than 12 times less than paraffin embedding. However, compared with the $x$-$y$ dimensions, the small depth of the slabs and the artificial edges of the $z$-planes could have had an influence on the shrinkage along the $z$-axis (cf. Ref. 4). Furthermore, it has always to be born in mind that different compartments might shrink differently (cf. Refs. 10, 11, 60). Therefore, the conversion into 3D has to be considered carefully. Nevertheless, the results indicate that shrinkage is most distinct during the applied protocol of paraffin embedding, followed by simple GMA embedding.

Shrinkage occurring in paraffin embedding is well known (e.g., Refs. 3, 14, 28, 36), but it has to be taken into consideration that the overall shrinkage is not only the result of the embedding medium but also the result of the effects of dehydration and intermediate agents. The work of Iwadare et al. (25), however, which demonstrates shrinkage of liver and kidney specimens during paraffin embedding, shows that shrinkage resulting from intermediate (xylene or chloroform) and liquid paraffin infiltration exceeds that resulting from dehydration in solvents (methanol or ethanol). In our study, however, after dehydration in acetone during GMA embedding, we found no further shrinkage, rather a slight swelling, after the next step. Therefore, our results support the notion that embedding in GMA is superior to paraffin embedding in terms of shrinkage and tissue deformation (10, 24, 38, 58). Dobrin (9) compared paraffin and GMA embedding on tissue dimensions of artery rings. Although he found a shrinkage

Table 2. Mean number of points on cut face per lung over all time points

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GA, glutaraldehyde; PFA, formaldehyde freshly prepared from depolymerized paraformaldehyde; GMA, glycol methacrylate; OsO4, osmium tetroxide; UA, uranyl acetate.

Table 1. Number of slabs investigated during embedding and evaluated

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Fig. 3. Illustration of cut face estimation by point counting. The black line frames the cut face of the slab, and the yellow areas indicate the areas per point hitting the cut face of the slab. The top right corner of each cross (indicated by the arrow on the left) is considered as infinitely small point for counting (see Refs. 23, 45, 53).

Shrinkage is a critical phenomenon occurring during processing of tissues for histology, especially when quantitative measurements have to be done (10, 60). Unless particle number is estimated by use of the physical dissector (47) in a fractionator design (19, 20), stereological estimates may severely be influenced by shrinkage, in particular estimates regarding size (10, 60). Shrinkage can influence quantitative measurements on two levels. On the one hand shrinkage may directly influence the stereological measurements on a section, since a shrunken and therefore smaller cell, for example, occupies a smaller area on a section leading to fewer points hitting the cell profile during point counting. Shrinkage might be acceptable if homogenous in the whole tissue, but once differential shrinkage of different compartments (perhaps even in a nonuniform distribution) occurs, even simple density measurements become biased (for review see Refs. 10, 60). Furthermore, even after homogenous shrinkage, later calculations of absolute values [conducted to avoid the “reference trap” (7)] may become biased if they include the total organ volume that had....

DISCUSSION

Shrinkage is a critical phenomenon occurring during processing of tissues for histology, especially when quantitative measurements have to be done (10, 60). Unless particle number is estimated by use of the physical dissector (47) in a fractionator design (19, 20), stereological estimates may severely be influenced by shrinkage, in particular estimates regarding size (10, 60). Shrinkage can influence quantitative measurements on two levels. On the one hand shrinkage may directly influence the stereological measurements on a section, since a shrunken and therefore smaller cell, for example, occupies a smaller area on a section leading to fewer points hitting the cell profile during point counting. Shrinkage might be acceptable if homogenous in the whole tissue, but once differential shrinkage of different compartments (perhaps even in a nonuniform distribution) occurs, even simple density measurements become biased (for review see Refs. 10, 60). Furthermore, even after homogenous shrinkage, later calculations of absolute values [conducted to avoid the “reference trap” (7)] may become biased if they include the total organ volume that had....

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during paraffin embedding, he actually found a volume expansion during GMA embedding (which was not significant compared with fresh tissue). He made use of the ability of GMA to be employed as a dehydrating agent (17, 18).

However, in our study, simple embedding in GMA resulted also in a considerable degree of shrinkage that could still severely distort stereological estimates. The use of OsO₄ and prolonged staining with UA before dehydration, however, reduces the overall shrinkage observed drastically to an almost negligible level. Indeed, the potential of OsO₄ and UA to reduce shrinkage during preparation of specimens for scanning electron microscopy, if used before dehydration, has been demonstrated previously (5). Regarding the lung, this experience has also been reported (38), and a study on the number of alveoli in the human lung (supplemental data in Ref. 40), in which some samples were monitored for shrinkage during embedding in GMA (Technovit 7100, after previous osmication but without UA), revealed no considerable dimensional changes. However, to our knowledge, no study has yet quantified this systematically for lung samples and compared it with simple GMA and paraffin embedding. As pointed out before, the use of OsO₄ and UA in morphometric studies of the lung appears advantageous because of their effects on lipids and membranes [and therefore on pulmonary surfactant (see Refs. 24, 38)]. In light of our findings regarding shrinkage, this impression is further supported.

Recently, Braber et al. (6) compared different fixatives, routes of application, and paraffin and plastic embedding of lung tissue of lipopolysaccharide-induced emphysema in terms of tissue morphology and emphasized good results after intratracheal instillation of formalin and embedding in paraffin. Furthermore, they compared mean linear intercept measurements (Lₘ) of the differently processed tissue. However, they did not quantify shrinkage during processing, and their protocols did not include GA, OsO₄, or UA. The latter, however, seem to contribute essentially to the preservation of original tissue dimensions during processing. Therefore, their use in quantitative studies {also those including Lₘ measurements (for detailed review of Lₘ, see Ref. 27)} should be taken into consideration. Results obtained from different experimental groups might indeed retain their comparative value, even if shrinkage occurred, but only if both groups shrank in the same fashion (consider fibrotic vs. emphysematous lungs) (24), a prerequisite that is hard to prove (cf. also Ref. 7).

One might ask whether osmication and UA also prevent shrinkage during the paraffin embedding protocol. The advantages of plastic embedding are the lesser degree of tissue deformation and the opportunity to make thinner sections (10), which per se are important arguments for the preferential use of plastics in solely morphometric studies (17) {for example, because of Holmes’ effect (53), cf. Fig. 8}. Furthermore, section thickness is more constant than it is in paraffin sections.
(17) (for a detailed discussion of the pros and cons of plastic embedding the reader is referred to Ref. 17). An advantage of paraffin embedding, however, is its long tradition and establishment of stains, including stains for immunohistochemistry (IHC), which might be a problem in plastic sections (10) [although they may be possible after some modifications, even enzyme activities may well be demonstrated (17)]. The use of OsO₄, however, might impair antigenicity and therefore reduce the success of immunohistochemical staining (1), thus negating one of the major advantages of paraffin embedding. Furthermore, it is not recommended for paraffin embedding (1). Therefore, the combination of OsO₄ and UA with paraffin instead of plastics appears less logical. However, regarding GMA embedding, it should be noted that OsO₄ might impair the polymerization process because of interactions with the GMA-monomer (16, 17). Nevertheless, a combination is possible and can result in excellent images, as demonstrated by Gerrits et al. (16) using Dalton’s fixative (containing OsO₄) for postfixation, ethanol/acetone dehydration, and Technovit 7100 embedding and Mühlfeld et al. (34) for the lung using OsO₄, UA, acetone, and Technovit 7100. Comparative micrographs of mouse lung sections after the three processing protocols of this study can be found in Fig. 8.

As stated above, several studies in the literature deal with the shrinkage of tissues after paraffin embedding. Fukaya and Martin (14) reported a mean area shrinkage of 17% after 4% formaldehyde fixation of degassed cat lungs, dehydration in methyl alcohol, and paraffin embedding. Lum and Mitzner (28) investigated shrinkage in 11 laboratory species and found a linear shrinkage of 20% in mouse lungs from fixed to embedded tissue after filling the lung with 10% neutral buffered formalin with a hydrostatic pressure of 25 cmH₂O. Our findings are in line with those of Lum and Mitzner (after conversion to one dimension by drawing the square root we also end up with roughly 20% shrinkage for paraffin embedding), whereas Fukaya and Martin found a lower degree of shrinkage. Lum and Mitzner have already pointed out that the difference might be because of the different types of fixation (degassed vs. filling with fixative) and the remaining recoil in the filled lungs. Our findings support this interpretation. Residual elastic recoil has also been suggested by Yan et al. (62) recently as a possible cause for the lower Cavalieri lung volume estimates they obtained compared with estimated volumes based on Scherle’s method. They referred to the work of Oldmixon et al. (43), who demonstrated that GA is unable to completely fix elastin, and to the work of Bachofen et al. (2), who demonstrated residual retractive forces after perfusion fixation with GA (cf. also Ref. 46). Boonstra et al. (3) reported a linear shrinkage of about 12.9% of tissue from the cervix uteri after dehydration, clearing, and embedding in paraffin. These lower values also support the theory of elastic recoil, since the cervix uteri lacks such an extensive network of elastic fibers as found in the lung (41). This could explain the lesser degree of shrinkage in their studies.

It has to be taken into account that the shrinkage values presented here only reflect the shrinkage during histological
processing and not the shrinkage because of fixation itself. Besides possible shrinkage during fixation itself (e.g., Refs. 3, 14, 15, 28, 31, 51, 57), the fixative seems to have an influence on the preservation of tissue dimensions during later processing. In addition to the better preservation of tissue dimensions by treatment of the samples with OsO₄ and UA before dehydration, the use of GA in combination with PFA seems to result in a further improvement compared with simple formalin fixation, although the effect is rather small. The results are in line with the common opinion that GA fixes tissues with more stability than formaldehyde (e.g., Ref. 58). One might argue that GA is unsuitable for histological studies, at least if immunohistochemical investigations are to be carried out, since GA may impair antigenicity more than formaldehyde (24). There is a simple workaround (50), however, allowing both IHC and the use of GA for light microscopical morphometric studies: initial fixation is carried out with an IHC-suitable fixative (e.g., only formaldehyde or a low concentrated GA-containing fixative, an example is given in Refs. 17, 24). Next, before further processing, the organ is sampled according to SUR sampling (see above), ending up with a SUR sample for IHC and another SUR sample for morphometry. Samples for morphometry can then be postfixed by immersion in a stronger GA fixative followed by treatment with OsO₄ and UA before further processing and therefore before the shrinking effects of dehydration and embedding can take place. Regarding the IHC, however, there may be some antigens that are not compatible with aldehyde fixation in general and with GA fixation in particular. This has to be tested for each specific case.

Fig. 7. Shrinkage during GMA embedding after postfixation with OsO₄ and UA before dehydration, mean per lung.

Fig. 8. Comparative micrographs of mouse lungs (fixed with 1.5% GA, 1.5% PFA in 0.15 M HEPES) processed according to the three different protocols of this study. Scale bar = 100 µm. A: paraffin (section thickness: 4 µm, hematoxylin and eosin staining). B: simple GMA embedding (1.5 µm, toluidine blue staining). C: GMA embedding after treatment with OsO₄ and UA before dehydration (1.5 µm, toluidine blue staining). Image acquisition was carried out using ×10 objective magnification and a Leica (Wetzlar, Germany) DM 6000 B microscope. The advantages of thin sections that can be produced from GMA blocks are clearly visible. The thicker paraffin section shows both tangentially sectioned bronchiolar wall as well as alveolar septal wall profiles projecting over each other, thus potentially influencing quantification because of overprojection (Holmes' effect; see text).
Besides chemical fixation, rapid freezing and subsequent freeze substitution (e.g., Ref. 31) may be an alternative fixation method. However, as demonstrated and discussed in detail by Weibel et al. (56), this is a less suitable fixation method for morphometric studies of the lung because of inhomogenous fixation within the same specimen and a rather low reproducibility, circumstances that interfere with the basic demand on tissue sampling for stereological studies, i.e., every part of the organ having the same chance of being sampled (e.g., Refs. 24, 42), and thus every part of the organ having to be adequately fixed (see also Ref. 39). Weibel and his coworkers’ observations even made them abandon an initial plan to carry out comparative morphometric studies (freeze substitution vs. perfusion fixation) (56).

In conclusion, the preservation of mouse lung tissue dimensions during processing for morphometric analyses can be improved by embedding the samples in GMA and treating them with OsO₄ and prolonged staining with UA before dehydration and embedding. Initial fixation with a mixture of GA and PFA can further improve the preservation of tissue dimensions compared with simple formalin fixation. For quantitative histological studies, fixative and embedding protocol should be chosen carefully. The present study suggests that GAPFA fixation followed by OsO₄ and UA postfixation and GMA embedding is the method of choice for preservation of original tissue dimensions of mouse lung during dehydration and embedding.

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DISCLOSURES

At the time of submission the authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Author contributions: J.P.S. and M.O. conception and design of research; J.P.S. and M.O. experimental design; J.P.S. performed experiments; J.P.S. analyzed data; J.P.S. and M.O. interpreted results of experiments; J.P.S. prepared figures; J.P.S. drafted manuscript; J.P.S. and M.O. edited and revised manuscript; J.P.S. and M.O. approved final version of manuscript.

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