An unbiased stereological method for efficiently quantifying the innervation of the heart and other organs based on total length estimations

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Mühlfeld C, Papadakis T, Kraseva G, Nyengaard JR, Hahn U, Kummer W. An unbiased stereological method for efficiently quantifying the innervation of the heart and other organs based on total length estimations. J Appl Physiol 108: 1402–1409, 2010. First published February 11, 2010; doi:10.1152/japplphysiol.01013.2009.—Quantitative information about the innervation is essential to analyze the structure-function relationships of organs. So far, there has been no unbiased stereological tool for this purpose. This study presents a new unbiased and efficient method to quantify the total length of axons in a given reference volume, illustrated on the left ventricle of the mouse heart. The method is based on the following steps: 1) estimation of the reference volume; 2) randomization of location and orientation using appropriate sampling techniques; 3) counting of nerve fiber profiles hit by a defined test area within an unbiased counting frame on paraffin sections stained immunohistochemically for protein gene product 9.5; 4) electron microscopic estimation of the mean number of axon profiles contained in one nerve fiber profile; 5) estimation of the degree of tissue shrinkage of specimens in paraffin; and 6) calculation of the total axon length within the reference volume, taking tissue shrinkage into account. In a set of five mouse hearts, the total length of axons ramifying between cardiomyocytes ranged between ~50 and 100 m, with a mean of 75.98 m (SD 23.73). The time required for the microscopical analysis was ~8 h/animal for an experienced observer. Using antibodies specific for different neuron subtypes and immunoelectron microscopy, this method is also suited to estimate the total axon length of neurons expressing different transmitters. This new and efficient method is particularly useful when structural remodeling takes place and is suspected to involve gain or loss of axons.

design-based stereology; electron microscopy; innervation; myocardium

Changes in the innervation of organs may involve functional characteristics (e.g., expression of a certain neurotransmitter) or structural features (e.g., gain or loss of axon branches). Such alterations are being considered to be of importance in a variety of human diseases in different organs. In the heart, for example, pressure-induced myocardial hypertrophy causes neuronal dysfunction and increased sympathetic innervation, as shown by qualitative immunohistochemistry (20). Diabetic cardiomyopathy is associated with initial downregulation of vasoactive intestinal peptide (7), intra-axonal accumulation of calcitonin gene-related peptide (4), as well as reduced uptake of scintigraphic tracers of the sympathetic cardiac innervation (13). In a rat model of heart failure, the injection of nerve growth factor into the stellate ganglion improved the re-uptake of norepinephrine in the heart (21), and transgenic mice overexpressing nerve growth factor are characterized by myocardial hyperinnervation and cardiac enlargement (12), with the latter possibly being mediated by a prosurvival activity of the growth factor (3). If structural changes of the innervation of the heart (or any other organ) are thought to occur, it is desirable to estimate the extent of these alterations in an accurate, precise, and biologically meaningful way.

In the present article, we address the scenario of gain (hyperinnervation) or loss (hypoinnervation) of axons in the heart. First-order quantitative characteristics to address structural changes are volume, surface area, length, and number as total values referred to a well-defined reference volume (e.g., the left ventricle of the heart) (33). It appears reasonable to assume that sprouting or degeneration will most likely affect the total length of axons or the total number of axon branches between two branching points in a given reference volume. Although the estimation of the total number of axon branches seems theoretically possible by estimating the connectivity of nerve fibers (11, 32), the estimation of total length promises to be a more efficient and equally meaningful parameter. According to stereological principles (reviewed in Ref. 29), the quantification of length is simply based on the counting of object profiles within an unbiased counting frame area (10). The relation of the number of object profiles multiplied by 2 to the area of the counting frames provides a length density, which needs to be converted to the total length of the objects by multiplication with the reference volume. A number of preconditions, however, have to be met. 1) As in all stereological studies, each part of the reference volume has to have an equal chance of being included into the analysis, which is guaranteed by an appropriate sampling design [e.g., systematic uniform random sampling (SURS), see Ref. 26]. 2) When surface area or length is estimated, the orientation of the tissue needs to be randomized by an appropriate method, such as the orientator (25) or the isector (31).

Estimation of the total length of nerve fibers has been performed, for example, in human brains in Alzheimer’s disease (19), but, in the peripheral nervous system, a number of further problems arise. 1) The visualization of nerve fibers in light microscopy requires the use of highly specific antibodies and the use of embedding protocols that guarantee successful immunohistochemistry of nerve fibers. Paraffin embedding, however, leads to an unpredictable tissue shrinkage, which even does not necessarily have the same extent in different experimental groups (6). 2) Stereology aims at obtaining three-dimensional information about objects from two-dimensional section planes. Keeping section thickness minimal guarantees that overprojection does not greatly influence the results from a stereological study. In case of nerve fibers, however, focusing...
through a light microscopic section provides essential information on whether the site of immunoreactivity really represents a nerve fiber profile. 3) At the light microscopic level, it cannot be identified how many axon profiles are contained in a single light microscopic nerve fiber profile. This may seriously underestimate the total innervation, but may also lead to false conclusions, if the pathological process that is looked at does only influence the number of axons within one light microscopic nerve fiber profile, but not the number of light microscopic nerve fiber profiles itself.

The present article provides an efficient method for quantifying the total length of axons in organs illustrated on the left ventricle of the mouse heart. To define a functionally coherent group of axons, the structure of interest was defined as those axons ramifying among the cardiomyocytes and capillaries, but not those axons associated with pre- or postcapillary blood vessels or larger nerve fiber bundles running in connective tissue septa. We demonstrate how the errors arising from the mentioned problems can be kept minimal and how the contribution of the method to the overall observed variability between individual subjects can be controlled.

MATERIALS AND METHODS

In the following, we describe the various steps of how to perform the quantification of axon length. A comprehensive illustration of the subsequent or parallel steps of this method is presented in Fig. 1. The description also refers to several stereological methods that are not explained in detail here. The reader may, for example, refer to Ref. 29 for further information and illustration.

Animals and tissue preparation. Five 7- to 9-wk-old female C57Bl6/J mice were used in this study. All mice were housed in individual cages, with free access to standard food and water in a room at 20–22°C, with a 12:12-h light-dark cycle. Euthanization of the mice was reported to the responsible authorities. The animals were part of a control group in a different study (8). The experimental protocols were approved by the Bioethical Committee of the District of Braunschweig, Germany. All of the experiments described in this study comply with the current German laws and conform to the Guide for the Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research, 1996.

Mice were killed by cervical dislocation, a median thoracotomy was performed, and the hearts were quickly excised and fixed in 4% paraformaldehyde in PBS (pH 7.35, 1.395 osmol/l). The hearts were stored in cold fixative for ~12 h. Afterwards, the left ventricle, including the interventricular septum, was separated from the rest of the heart, weighed, and cut along the longitudinal axis. From the mass of the left ventricle, the left ventricular volume was calculated by dividing the mass by the density of muscle tissue, 1.06 g/cm³ (27). The left ventricle was further sectioned three times transversally. From the tissue blocks, two were chosen by SURS for paraffin embedding and immunohistochemistry and estimation of the length density of nerve fibers at the light microscopic level. Two tissue blocks were immersed for 2 h in a fixative containing 1.5% glutaraldehyde, 1.5% paraformaldehyde, in 0.15 M HEPES buffer for transmission electron microscopy embedding, as described previously (28). In short, the tissue blocks were subsequently washed, immersed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, washed, incubated in half-saturated aqueous uranyl acetate, washed again, dehydrated in an ascending acetone series, and finally embedded in araldite (Serva, Heidelberg, Germany).

Randomization of location and orientation. A thin section from an organ used for quantification should be representative of the whole organ, i.e., it should be random with respect to location and provide each part of the organ an equal chance of being selected. From the whole organ level down to obtaining test fields from the microscopic sections, SURS was employed to provide truly representative parts of the left ventricle for further analysis. In SURS, the first item is chosen randomly, and, starting from that, all subsequent items are selected within a defined step length.

Stereological estimation of the length of an object also requires that every orientation of the object has an equal chance of entering the analysis. A useful approach to this is the use of isotropic uniform random specimens. The tissue blocks for light microscopy were embedded in a spherical agar mould with a diameter of 5 mm, then rolled upon a table, and picked up at a given stop point. The specimens were embedded in agar in this random position (31). The tissue blocks for electron microscopy were sectioned according to the orientator principle (25), providing an isotropic uniform random section plane.

Light microscopy. From the paraffin-embedded blocks, tissue sections (5 μm in thickness) were generated and stained for the panneuronal marker protein gene product (PGP) 9.5. In short, sections were deparaffinized using xylol and incubated with 1% H2O2 in methanol for 10 min, and demasking of epitopes was performed by microwaving in 10 mM citric acid (pH 6.0). After blocking of unpecific protein binding sites, sections were incubated with the primary antibody (rabbit polyclonal anti-PGP9.5 antibody, diluted 1:5,000, Biotrend, Köln, Germany) for 18 h. Sections were washed before incubation with the secondary antibody (peroxidase-linked donkey-anti-rabbit IgG, diluted 1:100, Amersham International Biotechnology, little Chalfont, Buckinghamshire, UK) for 1 h. After additional washes, visualization of the immunoreaction was performed by the 3,3’-diaminobenzidine reaction enhanced by 1.5% nickel ammonium sulphate. Sections were washed again, dehydrated, embedded in Eukitt (Sigma-Aldrich, Steinheim, Germany), and sealed with a coverslip.

The sections were investigated using an Olympus BX51 light microscope (Olympus, Hamburg, Germany) equipped with a digital camera, a computer, and the newCAST stereology software (Visiopharm, Denmark). Test fields were obtained by SURS, and an unbi-
ased counting frame with an area of 3,000 \( \mu m^2 \) was laid over the test fields. Using a step length of 200 \( \mu m \), a mean of 218 (SD 44) fields of view were sampled, providing a mean of 103 (30) nerve fiber profile counts for each animal. PGP9.5 positive nerve fiber profiles \( Q(nf) \) were counted, if they were inside the area of the counting frame and not hit by the left and bottom lines (exclusion lines) and their extensions (Fig. 2). Additionally, the number of counting frames used for this purpose was noted.

**Transmission electron microscopy.** From the araldite embedded tissue blocks, ultrathin sections (50- to 70-nm thick) were cut and mounted on formvar-coated copper support grids. Sections were stained with uranyl acetate and lead citrate and investigated with a LEO 902 transmission electron microscope (Zeiss, Oberkochen, Germany). The sections were scanned by a SIRS regime with very small step lengths to sample nerve fiber profiles. The edges of the fields of view were regarded as a counting frame, with the left and the lower border of the image representing exclusion lines. Every time a nerve fiber was observed that was not sectioned longitudinally and did not touch the exclusion lines, the number of axon profiles within the nerve fiber profile was counted (Fig. 3). Thus, when the nerve fiber was not fully within the field of view, the stage position had to be moved to make sure that the whole nerve fiber profile was used for counting. The need to exclude longitudinally sectioned nerve fibers arises from the theoretical consideration that axons belonging to the nerve fiber, but being in a tissue plane under or above of the section plane, cannot be counted. Although this is a very rare event (it happened only once in this study), the rule for exclusion should affect all nerve fibers with the same probability. One such approach that has been used here is that nerve fibers are regarded to be sectioned “longitudinally” when the longest diameter was at least five or more times longer than the smallest diameter. From these counts, the mean number of axon profiles per nerve fiber profile \( Q(axon/nf) \) was calculated.

**Tissue shrinkage.** In contrast to epoxy or glycol methacrylate resin embedding (6, 8), paraffin embedding leads to a significant tissue shrinkage, which should be considered, if densities are directly related to the paraffin-embedded reference volume. In this case, paraffin shrinkage, which tends to be unimportant (see e.g., Ref. 1).

An estimator for the total length of nerve fibers \( L(nf, ref) \) is subsequently obtained by multiplication with the reference volume \( V(ref) \).

\[
L(nf, ref) = 2 \times \sum Q(nf) \times [1 - d(shr)]^{2/3} \times V(ref)
\]

To estimate the total length of axons, one needs to know the total axon profile number \( Q(axons) \) on the evaluated counting frames. As explained in the Introduction, it is not possible to detect all axon profiles on the light microscopic image. Instead, the mean number \( Q(axons/nf) \) of axon profiles per nerve fiber profile was estimated from electron microscopic sections by SIRS, as described above. We may safely assume that the mouse heart is homogeneous with respect to this mean number. Moreover, the results from electron microscopy are unbiased and stochastically independent from the light microscopic counts. Thus the product \( Q(axons/nf) \times Q(nf) \) is an unbiased estimator for the total number of axon profiles in the counting frames. The resulting estimator for the total length of axons in the reference volume \( L(axons, ref) \) is given by

\[
L(axons, ref) = \frac{2 \times \sum Q(axons/nf) \times \sum Q(nf)}{\sum A(ref, CF)} \times [1 - d(shr)]^{2/3} \times V(ref) = Q(axons/nf) \times L(nf, ref)
\]

**Preembedding for correlative light and electron microscopy.** One mouse heart was fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer for 16 h and, after washing in buffer, immersed in 18% sucrose in 0.1 M phosphate buffer for 16 h. Afterwards, the heart was frozen in liquid nitrogen and cut using a cryocutmicrome. Thus free-floating 40-\( \mu m \) thick sections were obtained. The sections were stained for PGP9.5 using the same protocol as described in the section Light microscopy above. Afterwards, the sections were osmicated, contrasted with half-saturated uranyl acetate, dehydrated in an ascend-
ing ethanol series, and finally flatly embedded in epoxy resin. The embedded section was viewed under a light microscope, and a region of interest was chosen, cut out of the section, and glued on a resin blind block. Afterwards, consecutive semi- and ultrathin sections were cut. Using light and electron microscopy, the nerve fibers stained for PGP9.5 could be analyzed at both magnification levels.

Statistical analysis. The estimates of the total length of axons from the set of mouse hearts analyzed in this study vary around the mean, with a certain degree of variation, which can be expressed by the coefficient of variation (SD/mean). Two sources contribute to the observed variation in the results: first, the total axon length differs individually from animal to animal, and, second, the stereological estimates of these lengths are subjected to statistical variation (error).

The statistical estimation error, expressed as squared coefficient of error (\(CE^2 = \text{variance/mean}^2\)), is calculated individually for each animal. The error coefficients of the estimators for nerve fiber length density and total nerve fiber length are identical, since \(L(nf,ref)\) is obtained from \(L(V(nf/ref))\) by multiplication with a constant factor. A shortcut formula for the error coefficient is

\[
CE^2[L(nf, ref)] = CE^2 \left( \frac{\sum Q}{\sum A} \right) = CE^2 \left( \frac{\sum Q^2}{\sum A} - \frac{1}{n_{CF}} \left( \frac{\sum A^2 - 2 \sum A \sum Q}{\sum A^2 A} \right) \right)
\]

where \(n_{CF}\) denotes the number of counting fields. This formula can be found in Ref. 22. It is based, first, on an approximation for the \(CE^2\) of a ratio of two random variables \(X\) and \(Y\), with correlation coefficient \(r_{XY}\) (see, e.g., Ref. 5),

\[
CE^2 \left( \frac{X}{Y} \right) = CE^2(X) + CE^2(Y) - 2r_{XY}CE(X) \times CE(Y)
\]

and, second, on the formula for the \(CE^2\) of a sum of independent, identically distributed random variables \(X_1, \ldots, X_n\),

\[
CE^2 \left( \sum_{i=1}^{n} X_i \right) = \frac{1}{n} NE^2 X
\]

The error coefficient of the mean number of axons per nerve fiber is obtained from the variance \(\text{var}[Q(axons)]\) of axon per nerve fiber counts as

\[
CE^2[Q(axons/nf)] = \frac{1}{n_{nf}} \times \frac{\text{var}[Q(axons)]}{Q(axons,nf)^2}
\]

where \(n_{nf}\) is the number of nerve fibers used in the determination of \(Q(axons/nf)\).

From this, the error coefficient of the product \(L(axons,ref) = Q(axons/nf) \times L(nf, ref)\) is calculated as

\[
CE^2[L(axons, ref)] = CE^2[Q(axons,nf)] + CE^2[L(nf,ref)]
\]

The latter formula makes use of the stochastic independence of the two estimators \(L(axons,ref)\) and \(Q(axons/nf)\).
Table 1. Observed and biological variation, coefficient of error

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CV (obs)</th>
<th>CE (meth)</th>
<th>CV (bio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L(nf/ref)</td>
<td>0.253</td>
<td>0.118</td>
<td>0.224</td>
</tr>
<tr>
<td>Qd(axons/nf)</td>
<td>0.171</td>
<td>0.209</td>
<td>*</td>
</tr>
<tr>
<td>L(axons, ref)</td>
<td>0.312</td>
<td>0.241</td>
<td>0.198</td>
</tr>
</tbody>
</table>

Note. *As depicted by Figure 4, very few nerve fibres contained a larger number of axon profiles, thereby increasing the CE (meth). If CE (meth) is larger than CV (obs), the CV (bio) cannot be calculated. Abbreviations: CV (obs) = observed coefficient of variation, CE (meth) = coefficient of error of the method, CV (bio) = biological coefficient of variation, L(nf/ref) = length density of nerve fibres, Qd(axons, nf) = mean number of axon profiles per nerve fibre profile, L(axons, ref) = total length of axons in the reference volume.

Since the stereological estimators are virtually unbiased, the total observed variance (varobs) of estimated lengths can be explained as the sum of biological variance (varbiol) and the mean variance (varmeth) due to the stereological estimation. In terms of error coefficients, we can, therefore, express the biological variation as

$$CV_{biol}^2 = CV_{obs}^2 - CE_{meth}^2$$

where CE$_{meth}^2$ is the mean variance within animals due to the statistical error of the stereological estimation, divided by the squared mean of the estimates over all animals. For a total of $m$ animals, we have

$$CE_{meth}^2 = \frac{1}{m} \sum \text{var}_{meth} = \frac{1}{m} \sum (CE^2 \times \text{estimate}^2) = \frac{1}{m} \sum \text{estimate}^2$$

(12)

Negative values for CV$_{biol}^2$ are possible, since Eq. 11 only provides a statistical estimator for the biological CV. This happens frequently if the error due to stereological estimation dominates the observed variation. Table 1 summarizes the results of the CE analysis for the five animals comprising the study.

RESULTS

At the light microscopic level, on average, a total of 212 (SD 41) counting frames hitting myocardium were studied per animal, yielding randomly 103 (SD 27) counts of nerve fiber profiles. Tissue shrinkage in paraffin was 42.4% (SD 13.1). The mean number of axon profiles per nerve fiber profile ranged between 2.15 and 3.48 with a mean of 3.04 (SD 0.52). Figure 4 shows the relative frequency of nerve fiber profiles with a specific number of axon profiles. In the five left ventricles of mouse hearts, the total myocardial axon length ranged between ~50 and 100 m, with a mean of 75.98 m (SD 23.73). Figure 5 demonstrates the individual values for each animal for nerve fiber length (only light microscopic analysis), for nerve fiber length after correction of tissue shrinkage, and the final values for axon length (combining light and electron microscopy and correction for tissue shrinkage). Correlative light and electron microscopy demonstrated that single axons are detected by light microscopy (Fig. 6).

DISCUSSION

The present study provides an unbiased and efficient method to estimate the total length of axons in solid organs. The application of this method to the left ventricle of the mouse heart reveals a total length of ~75 m of axons supplying cardiomyocytes and their direct microenvironment. In principle, this method can be applied to a variety of organs and scenarios, particularly those where sprouting or degeneration of axons is thought to occur.

Given the small size of the mouse left ventricle, the absolute value of 75 m appears unexpectedly high. However, a plausibility check can be performed, based on the geometric consideration of a tube with a diameter of 0.5 μm and a length of 75 m. The volume of such a tube can be calculated from $V = \pi * (0.25 \mu m)^2 * 75 m = 0.01473 mm^3$. In a mouse left ventricle of 90 mm$^3$, this would account for a volume fraction of 0.016%, which seems to be in a realistic range.

Based on the principles of design-based stereology, length estimation requires counting the number of object profiles in a defined area and multiplication of length density with the reference volume. Similar approaches were performed on myelinated nerve fibers in the brain (19, 34), unmyelinated fibers in the brain (24), but also on cholinergic nerve fibers in the smooth muscle of pig urinary bladder (30). The estimation of myelinated fiber length in the brain is based on light microscopy, since each fiber profile provides exactly one axon profile. In contrast, estimation of unmyelinated fiber length requires the higher resolution of transmission electron microscopy. Obviously, it would be possible to estimate the length density of axons in solid organs directly from transmission microscopy gathered from both light and electron microscopy, thereby providing a suitable mean to quantify cardiac innervation accurately.
electron microscopy sections as well. However, in contrast to
the brain, this would require a vast amount of tissue blocks
(and time) to provide a similar precision as provided by
counting immunoreactive nerve fiber profiles in the light mi-
croscope. The combination of light and electron microscopy in
the present study is, therefore, a far more efficient approach for
organs. Nielsen et al. (30) used only light microscopy to
estimate the length of cholinergic nerve fibers in the urinary
bladder, which underestimates the total length of axons and
leaves changes that occur within one nerve fiber profile unex-
plored. If there are no changes of the number of axons per
immunoreactive profile, this underestimation may be accepted
for between-group comparisons. However, physiological or
pathological alterations may not become evident at the light
microscopic level only.

There are a number of factors that may influence the results
obtained by our method that need to be considered. In nearly
every microscopic study, the fraction of tissue that is looked at
in the microscope is very small compared with the organ, of
which the study is thought to be representative. Rigorous
sampling using established methods is, therefore, necessary
(26). In the present study, only two tissue blocks were used
each for light and electron microscopy, providing a sufficient
precision. For larger hearts, e.g., human or pig hearts, the
number of sections needs to be increased because of the
heterogeneity of the innervation within the ventricular wall,
i.e., a transmural gradient with a higher sympathetic nerve fiber
density in the subepicardial layers than in the subendocardial
layers or a gradient from the apex to the base of the ventricle
(16). Length estimation requires that the tissue sections are
isotropic uniform random, which means that every orientation
of the tissue has an equal chance of being analyzed. Both the
orientator (25) and the isector (31) provide simple and efficient
ways to guarantee isotropic uniform random tissue sections.

Tissue shrinkage may seriously affect the results of a stereo-
logical study, if it occurs during tissue processing after esti-
mation of the reference volume (6). The present study confirms
a high degree of tissue shrinkage in paraffin embedding, which
is in good agreement with the literature (17). This shrinkage
should be considered when the final results are reported.
Importantly, tissue shrinkage may differ between experimental
groups, for example, due to different content of water (14).
For this method, it is only important to correct for tissue shrinkage
in paraffin embedding, but not in epoxy resin embedding,
because the mean number of axon profiles per nerve fiber
profile is independent of the tissue shrinkage.

The use of antibodies for quantitative studies is always a
critical point and raises concerns about antibody specificity and
labeling efficiency. Of course, the specificity of antibodies
needs to be checked using appropriate controls (no primary
antibody controls, preabsorption, knockout animals). In this
study, nerve fibers were detected by their immunoreactivity for
the panneuronal marker PGP9.5 (9, 18) for which a well-
characterized antibody with high specificity was used. Addi-

Fig. 6. Correlative light and electron microscopy
of single axons stained for PGP9.5. A, B, and D
are taken from an ultrathin section by electron
microscopy; D is a digital magnification of a
small region of interest taken from the following
semithin section by light microscopy. At higher
magnification, two nerve fibers were identified
that consisted of two (A) or one (B) stained
axons (marked by arrows). At light microscopic
level, the same region of interest was identified
(C) and compared with the low-power electron
micrograph shown in D. Thus it is demonstrated
that single-axon nerve fibers can indeed be vi-
sualized by light microscopy, but also that it is
impossible to say at light microscopic level
whether a nerve fiber contains one or more
axons.
tionally, morphological information helps to distinguish between sites of artifactual immunoreactivity product (e.g., insufficiently blocked endogenous peroxidase activity). Therefore, the greater problem seems to be labeling efficiency. For example, fixation and embedding protocols, including duration of fixation and concentration of fixation agents, may influence the degree of immunostaining. In the beginning, we had performed fluorescence staining on frozen sections, but did not find substantial differences in the immunoreactivity or visibility of the PGP9.5-labeled nerve fibers. Nevertheless, depending on the investigator’s preference or the suitability within a study design to use paraffin-embedded or frozen tissue, we recommend the strict application of standardized protocols, especially between experimental groups. This does not solve the problem of different protocols influencing the total length estimation, but it keeps the differences in labeling efficiency within one study minimal.

Overprojection influences stereological volume and length estimations, depending on the thickness of the section. Usually, the influence is larger in volume estimations, and a rule of thumb is to work with section thicknesses of approximately one-tenth the size of the structure (15). In the light microscopic analyses presented here, it was not possible to apply this rule. Besides technical reasons (sectioning), the spatial information from a 5-μm thick section is needed to distinguish small precipitations of immunoreactive product from nerve fiber profiles by focusing through the section. Due to the small focal depth of ×40 lenses, it seems that the overprojection effect is kept minimal, if only objects in focus in a defined focal plane are counted. We propose the following procedure: the top and the bottom of the section are determined for each test field, and the focal plane in the middle of the section is used in each test field.

One crucial aspect of the method is that the probability of a nerve fiber to be sampled at the light microscopic level is the same as at the electron microscopic level. Two potential scenarios were identified to be of particular importance. First, can nerve fibers containing one axon only be identified by light microscopy? Second, is it possible to clearly identify nerve fibers at the electron microscopic level solely based on their morphology? Both questions were evaluated in a couple of qualitative preembedding experiments, with sections being labeled by PGP9.5 and the immunoreactivity being visualized by the 3,3′-diaminobenzidine reaction (Fig. 6). Thus we were able to correlate the counting events obtained from light and electron microscopy with each other by their immunoreactivity. Importantly, we did not observe labeled nerve fibers at the electron microscopic level that could not have been identified at the light microscopic level. However, it must be noted that the identification of axon profiles between the myocytes requires extensive experience with the interpretation of transmission electron microscopy of the heart and the peripheral nervous system. It may, therefore, be useful to look at a number of immunostainings of axons in the electron microscope before starting to count. If the identification of the axons, solely based on morphological criteria, remains difficult, the use of immunoelectron microscopy as a routine identification method is recommended. Although this may sound very labor intensive at first glance, our results clearly show that the number of nerve fiber profiles counted with light microscope only definitely underestimates the mean number of axons within one nerve fiber profile. If subpopulations of axons (containing specific transmitters, for example) are to be studied, immunoelectron microscopy has to be applied anyway.

Both the nerve fiber counts from light microscopy and the axon counts from electron microscopy contribute equally to the variation of the resulting estimates for the total axon length, as manifested by Eq. 10. An optimal allocation of workload should, therefore, strive to obtain roughly equal CE for both L(ν(nf/ ref)) [or L(ν(nf/ ref))] and Q0(ν(axons/nf)).

It can be seen from Eqs. 6–9 that the CE2 is inversely proportional to the number of evaluated fields or nerve fibers. In the present situation, we found CE2ό[Q0(ν(axons/nf))] to be higher than CE2ό[L(ν(nf/ ref)], which means that any additional work should rather be invested in increasing the precision of Q0(ν(axons/nf)) than in improving on CE2ό[L(ν(nf/ ref)].

One has to be aware, though, that Eq. 6 (and Eq. 8) refers to the case of independent random samples, while the microscopic sections were actually evaluated using a systematic sampling design. Systematic sampling reduces the estimation variance, if the tissue is inhomogeneous, e.g., if it consists of regions with higher and lower innervation. This means that the formulas for the estimation of the CE tend to overestimate the true variability.

For an experienced observer, the total amount of time needed to estimate the total length of axons in the mouse heart was ~8 h, not including the time for laboratory work: 3 h for counting of nerve fiber profiles at light microscopic level (1.5 h per tissue block), 4 h for counting of axon profiles at electron microscopic level (2 h per tissue block), and 1 h for estimation of tissue shrinkage. With 103 (SD 27) counting events at the light microscopic level, our pilot study was rather meant to demonstrate the way to implement the method than to provide the most precise estimates. It seemed important to show that, even at a relatively low number of counting events, the variability of the light microscopic estimates was not dominated by the counting procedure, and that it is rather important to put more work into the electron microscopic analysis (see paragraph above). Due to the mentioned transmural gradient of innervation (with a denser innervation in the subepicardial regions), the impact of heterogeneity becomes more important in larger organs. Depending on the required precision of the estimates, it is, therefore, likely to be necessary to include a greater number of samples into the analysis, which will increase the amount of time needed.

In summary, we present an unbiased stereological approach to estimate the total length of axons within a solid organ. We suggest that studies commenting on the “amount” of nerve fibers (i.e., hyperinnervation or degeneration) in solid organs should make use of this quantitative approach, as this allows the statistical comparison between experimental groups and provides a higher degree of comparability between different studies.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).
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