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DESIGN-BASED STEREOLOGY OF THE HUMAN HEART

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ABSTRACT

Design-based stereological methods for obtaining estimates of the sizes of myocardial fibers and the number and sizes of capillaries and myocardial fiber nuclei in human autopsy hearts are presented. Sampling of heart tissue was uniform random at all levels, also with respect to orientations for estimates of length and mean sizes as required. Global structural quantities were estimated as: total quantity : = density × volume of left ventricle. Ordinary, unbiased stereological methods were used for estimating the volume, surface area and length density of capillaries and myocardial fibers. The numerical density of fiber nuclei and capillaries were estimated using the optical disector and the physical disector, respectively. Local quantities ("size") were estimated either directly using unbiased estimators to obtain the average individual size and size distribution parameters or indirectly using the relationship: average size := total quantity / total number. As an individual myocyte cannot be distinguished in the sections used in our studies and may be polynucleated neither individual nor average myocyte volume can be estimated unbiasedly. Several efficient and robust alternatives are, however, provided for reporting various mathematically well defined aspects of fiber size.

Key words: capillary, fiber nucleus, human heart, left ventricle, myocardial fiber, stereology.

INTRODUCTION

This study describes design-based, unbiased stereological principles for estimating structural quantities of muscle fibers and capillaries of the human (autopsy) heart. In order to obtain unbiased estimates, uniform random sampling performed on the whole heart must be carried out at all the levels, i.e., slabs, blocks, sections, fields of vision, and orientations in 3D whenever necessary. The results of global parameters must be expressed as total quantities, in contrast to densities, in order to be interpreted unambiguously. The 3D sizes should be estimated using assumptions-free methods in order to provide useful information for the mechanisms of structural and functional changes.

The following notes are based on a larger study (Tang et al. 1999) and interested readers are referred to this manuscript for further details.

MATERIAL AND METHODS

The material comprised 6 hearts, from 4 female and 2 male autopsy cases, average age 36 years (range 18 – 50 years), average height 168 cm (range 154 – 177 cm), and average body weight 67 kg (range 62 – 73 kg). Average heart weight was 299 g (range 225 –335 g). The thickness of left ventricular wall was from 10 to 11 mm. The deaths of the patients whose hearts were used in this study were due to either accident, suicide, or homicide. Excluded were all individuals with sign of atherosclerosis, that is significant luminal narrowing of the coronary arteries, and patients with records of any pathological findings in the hearts. The complete hearts were immersion fixed in 0.1 M sodium phosphate buffered formaldehyde (pH = 7.2, 4% formaldehyde) for at least two months. The postmortem fixation delay of the hearts was about 2 days.

METHODS

(1) Estimation of the reference volume

The left ventricle and the interventricular septum were separated from the right ventricle and the atria. The left ventricle was embedded in 7% agar, and serially sectioned by a slicing machine, illustrated in Figure 24 in Gundersen & Jensen (1987), to provide between 10 to 15 slices of 10 mm thickness from left ventricle, with a random starting point. A transparent counting grid was placed at random over the cut surface of every ventricular slice. The left ventricular volume was calculated from Cavalieri's principle:

$$\mathbf{V(LV) := \bar{t} \times (a/p) \times \Sigma P(LV)} \qquad (1)$$

where ':= 'indicates that the result is the estimate value rather than the true value, **V(LV)** is the total volume of the left ventricle, $\bar{t}$=10 mm is the average slice thickness, **(a/p)** = 56 mm^2 is the area associated with each point in the grid, and Σ**P(LV)** is the total number of points hitting the left ventricle. On an average, 250 points were counted per left ventricle with a mean coefficient of error, CE[V(LV)], of 0.04 (Gundersen et al., 1998).

(2) Sampling and histological preparation of tissue

From the tissue slices, every second slice was systematically sampled, the first one being chosen at random. 2D isotropic, uniform random (IUR) square grid with small quadrates of 10 mm × 10 mm was superimposed at random on the slices sampled. For a 2D systematic fraction of points hidden by tissue under orthogonal projection the corresponding cube is cut using a ruler on top and a scalpel. The IUR sections were made using the orientator (Mattfeldt et al., 1990). From each sampled plastic block, two consecutive sections with a thickness of 2 μm, two **physical disector sections**, were cut, starting at least 5 mm below the surface of the block, and stained with periodic acid methenamine silver (PAMS) for the estimation of capillary number. One of the disector sections was used to estimate the total length, surface area, and volume of capillaries. A further 40-μm-thick section, an **optical disector section**, was cut and stained with Mayer's Haematoxylin and 0.15% Basic Fuchsine for estimating the number of the

nuclei of the myocardial fibers and connective tissues. One **thin section** of 2-μm -thickness was cut and stained with the modified azan staining procedure for estimating the sizes of the myocardial fibers and fiber nuclei.

(3) Estimation of total capillary length, volume, and surface area

The IUR sections mentioned above, i.e., one of the **physical disector sections**, were viewed using a modified Olympus BH-2 microscope with a PC and a monitor connected to a video color camera mounted on top of the microscope. By means of the CAST-Grid software (Olympus, Denmark), the stereological probes (points, lines and counting frames) were superimposed onto the video images of the tissue sections viewed on the monitor. An oil objective lens (60 X, NA 1.40) and an intermediate lens (2.5X) before the video camera were used when counting. Using a final magnification of 1400X, fields were systematically randomly sampled in the sections using a fixed x and y step length (1000 μm and 1000 μm, respectively) accomplished by a motorized specimen stage controlled by the program. The fields of vision where frames hitting artificial edges of the uniformly positioned sections were not used for counting.

The total length of the capillaries in the left ventricle, **L(cap,LV)**, was estimated as:

$$\mathbf{L(cap,LV) := L_V(cap/LV) \cdot V(LV) := \frac{2 \cdot \sum Q(cap)}{(a/p) \cdot \sum P(LV)} \cdot V(LV)} \qquad (2)$$

where $\sum$**Q (cap)** denotes the total number of capillary profiles which were counted in the unbiased 2D counting frame, within the sampled area of the left ventricle, **(a/p)** is a (frame) of 3000 μm^2 divided by 4 corner points, *p.* **a(frame)** = 3000 μm^2 is the area of each unbiased counting frame at the tissue level, **p** = 4 is the points used to estimate the reference area when the natural edge was met. $\sum$**P(LV)** is the total points hitting the reference space. On average, 220 profiles in about of 100 fields of vision were counted per left ventricle with a mean coefficient of error on the estimated total capillary length, CE[L(cap,LV)], of 0.13.

The total volume of the capillary lumina in the left ventricle, **V(cap,LV),** was estimated as :

$$\mathbf{V(cap,LV) := V_V(cap/LV) \cdot V(LV) := \frac{P'}{P} \cdot \frac{\sum P'(cap)}{\sum P(LV)} \cdot V(LV)} \qquad (3)$$

where $\sum$**P'(cap)** is the total number of points hitting capillary lumina within left ventricle, and $\sum$**P(LV)** is the total number of points hitting left ventricle. **P'/P** is the ratio of (line-end) points and (frame-corner) points in the test system. A ratio of 6:1 was used. On average, 100 points hitting capillary lumens per left ventricle were counted with a mean CE of the estimate of the total capillary volume, CE[V(cap,LV)], of 0.11.

The total luminal surface of the capillaries in the left ventricle, **S(cap,LV)**, was estimated as:

$$\mathbf{S(cap,LV) := S_V(cap/LV) \cdot V(LV) := \frac{2 \cdot \sum I(cap)}{l(p) \cdot \sum P(LV)} \cdot V(LV)} \qquad (4)$$

where $\sum$**I(cap)** denotes total number of intersections between the test lines and capillary luminal surface, $\sum$**P(LV)** total number of test points (at the ends of test lines) which hit the tissues, and **l(p)** the total length of test lines divided by all the number of test points used. On average, 400 intersections were counted per left ventricle with a mean CE of the estimate of total capillary surface area, CE[S(cap, LV)], of 0.09.

(4) Estimation of total capillary number

First of all, in order to estimate the number of capillaries, a single capillary should be unambiguously defined. The second point to be made is how to estimate the number of capillaries. In order to describe a three dimensional network quantitatively, topological concepts must be employed. That is, two topological properties: the number of disconnected parts of the capillary network and the redundant or extra connection, were estimated. The Euler-Poincare characteristic or Euler number, χ, of a complete capillary network is defined as:

$$\chi := \#\textbf{disconnected nets} - \#\textbf{ redundant connections} \qquad (5)$$

where #(·) means number of (·). The number of redundant connections is called the connectivity. Since the capillaries of a human heart is multiply connected in a highly complex network the number of disconnected networks is one, and the simplified relationship used here may therefore be reduced to:

$$\chi := \textbf{1- connectivity} \qquad (6)$$

Considering that connectivity is of the order 10^9, both the connectivity and the Euler number has a one-to-one relationship to the number of capillaries. The estimation of capillary connectivity was based on the physical disector principle (Sterio, 1984; Nyengaard and Marcussen, 1993). That is, two sections separated by a known distance are used. Events observed in the first section, but not in the second, are counted. The distance between the sections must be less than the smallest height (diameter) of the objects (capillary lumina) to be observed. The greater the distance between the sections, the more events are observed and the efficiency is enhanced. On the other hand, increasing the distance between the sections will make it more difficult to identify the objects in question, i.e., it is difficult to know whether it is a new capillary or it is different profiles of the same capillary changing position in three dimensions. The images of the two sections chosen were projected onto a table by means of two projection microscopes. Two 100X oil immersion objectives were used to obtain a magnification of 1650X. The Euler number, χ***(cap)***, was estimated by comparing the luminal events of capillaries sampled by means of the 2D unbiased counting frame in one section with

the corresponding capillaries in the next section. In detail, three 2D topological events were observed: 1) the appearance of a capillary lumen, implying an 'island', 2) the (rare) appearance of an isolated profile of capillary wall inside the lumen of capillary profile, implying a 2D luminal 'hole', and 3) the division of a simple capillary lumen into two (or more) capillary lumina, implying one (or more) 'bridges'. In estimating the Euler number contribution from the disector the surplus luminal bridges were calculated as:

$$\chi\,\mathbf{(cap)} := \Sigma\,\mathbf{islands} + \Sigma\,\mathbf{holes} - \Sigma\,\mathbf{bridges} \qquad (7)$$

The intuitive reason for this strict mathematical relationship is that each capillary bend that happens to look like a connection also creates an isolated island. The same is true for internal luminal holes.

The total number of capillaries per left ventricle, **W(cap, LV)**, was estimated by multiplying the capillary numerical density, $\mathbf{W_V(cap/LV)}$, with the left ventricular volume, **V(LV)**.

$$\mathbf{W(cap,LV)} := \mathbf{W_V(cap/LV)} \cdot \mathbf{V(LV)} := \frac{-\Sigma\chi\mathbf{(cap)}}{\mathbf{2 \cdot h} \cdot \Sigma\mathbf{a(LV)}} \cdot \mathbf{V(LV)} \qquad (8)$$

Where $\Sigma\chi$ **(cap)** is the Euler number of the capillaries, Σ**a(LV)** is the total area of the unbiased counting frames, and **h** = 2 μm is the height of the disector, equal to the section thickness. The (Euler) number of capillaries is precisely the number of new connections (not containing the very first one) that have to be generated to create the capillary bed present. The counting of the Euler number was performed in both directions so that all topological events of capillary lumina between the two physical section planes were noted, i.e. the disector was used from top to bottom and vice versa. Therefore, the height of a disector with topological events noted in both directions was **2h**. On average, 170 topological events of capillaries were counted per left ventricle with a mean CE [W(cap, LV)] of 0.10 (Gundersen et al., 1993).

(5) Estimation of average capillary length, surface area, volume, diameter, and related quantities

These values were all calculated from the above total estimates. The mean length of capillaries, $\bar{\ell}$ ***(cap)***, was estimated as:

$$\bar{\ell} := \frac{\mathbf{L(cap,LV)}}{\mathbf{W(cap,LV)}} \qquad (9)$$

Whereas the mean surface area of capillaries, $\bar{s}$ **(cap),** was obtained from

$$\bar{\mathbf{s}}\mathbf{(cap)} := \frac{\mathbf{S(cap,LV)}}{\mathbf{W(cap,LV)}} \qquad (10)$$

The mean luminal circumference of capillaries, $\bar{b}$ **(cap)**, was obtained from:

$$\bar{b}(cap) := \frac{S(cap,LV)}{L(cap,LV)} \tag{11}$$

The mean luminal cross-sectional area of capillaries, $\bar{a}$ **(cap)**, was obtained from the following equation:

$$\bar{a}(cap) := \frac{V(cap,LV)}{L(cap,LV)} \tag{12}$$

None of the above estimates make any assumption about capillary geometry except that its size in one dimension is much larger than in the other two.

Under the assumption that capillaries are circular cylinders the mean luminal diameter of capillaries, $\bar{d}$ **(cap)**, was derived from:

$$\bar{d}(cap) := \frac{S(cap)}{\pi \cdot L(cap)} \tag{13}$$

The average cross-sectional area of a 'typical' tissue sheet around a 'typical' capillary, $\bar{a}$ **(tiss/cap)**, was calculated as:

$$\bar{a}(tiss/cap) := \frac{1}{L_V(cap/LV)} := \frac{V(LV)}{L(cap,LV)} \tag{14}$$

The radius of the tissue around a 'typical' capillary, which may be interpreted as average maximal diffusion distance from capillary to tissue, $\bar{r}$ **(tiss/cap)**, was obtained by:

$$\bar{r}(tiss/cap) := \sqrt{\frac{\bar{a}(tiss/cap)}{\pi}} := \sqrt{\frac{1}{\pi \cdot L_V(cap/LV)}} \tag{15}$$

The mean tissue volume supplied by one capillary, $\bar{v}$ **(tiss/cap)**, was estimated from:

$$\bar{v}(tissue/cap) := \frac{1}{W_V(cap/LV)} := \frac{V(LV)}{W(cap,LV)} \tag{16}$$

It is worthy of notice that the above estimates of local size except $\bar{d}(cap)$ are independent of any shape assumptions, except that capillaries are very long compared to their diameter. Their cross sections may have any shape and their axes may be curved or twisted in any way.

(6) Estimation of the total number of fiber nuclei

The nuclei were defined as fiber nuclei if they were located inside the muscle fiber. All other nuclei were defined as endothelium and connective tissue nuclei. The fields of vision in each section were systematically randomly sampled. At a final magnification of 2400X, nuclei were sampled in each sampled field using the optical disector principle to obtain an estimate of the numerical density, N_V , of nuclei. The distance between the first and last optical section was 15 μm, which was read off on the electronic microcator. At each sampled field of view, the sampling volume was started 5μm below the upper surface of the section so that in a 40-μm-thick section a guard volume with a height of 20 μm could be established below the sampling volume. The microscope was focused down through a section and a nucleus was counted when it first came into focus and did not touch any exclusion lines in that focal plane. The total number of fiber nuclei, ***N(fibnuc,LV)***, was obtained from:

$$\begin{aligned} \mathbf{N(nuclei,LV)} &:= \mathbf{N_V(fibnuc/fib)} \cdot \mathbf{V(fib)} \\ &:= \frac{\sum \mathbf{Q^-(fibnuc)}}{\sum \mathbf{P(fib)}} \cdot \left[\frac{\mathbf{p}}{\mathbf{h} \cdot \mathbf{a}}\right] \cdot \mathbf{V_V(fib/LV)} \cdot \mathbf{V(LV)} \end{aligned} \qquad (17)$$

Where $\sum \mathbf{Q^-(fibnuc)}$ is the total number of fiber nuclei counted in all disectors per left ventricle, $\sum \mathbf{P\ (fib)}$ is the total number of points falling on the fibers in the ventricular tissue, **p** = 4 is the points which were used to count the points hitting the muscle fibers, **a** is the area of the test frame, and **h** is the height of the disector, the distance between the first and the last optical sections. An unbiased counting frame with an area of 1100 μm^2 was used to count the number of the fiber nuclei, and a larger one, with an area of 2700 μm^2 was used to count the number of the connective tissue nuclei. On average, 133 fiber nuclei and 106 connective tissue nuclei were counted per left ventricle with a mean CE[N(fibnuc, LV)] and CE[N(ctnuc, LV)] of 0.09 and 0.12, respectively.

(7) Estimation of the sizes of fiber nuclei

The IUR thin sections mentioned above were used to estimate the sizes of fiber nuclei. A 60X oil objective lens was used when measuring. Using a final magnification of 1400X, fields were systematically randomly sampled in the sections using a fixed x- and y-step length (900 μm and 900 μm, respectively) accomplished by a motorized specimen stage controlled by the computer program. An integral test system, with points and lines, was superimposed on the sampled images. The nuclear volume fraction, V_V(nuclei/LV), was estimated by point counting in the same way as the estimates of the capillary volume density. The total volumes of nuclei was estimated by multiplying V_V(nuclei/LV) with V(LV). The volume-weighted mean volume of fiber nuclei was obtained by the use of the stereological method of point-sampled intercepts (Gundersen and Jensen, 1985). For each point in the test system hitting the profile of a fiber nucleus in focus, the intercept length through the nucleus was measured from the nuclear border to the nuclear border. The intercept lengths were individually cubed. The mean volume-weighted volume of the fiber nuclei, $\bar{v}\mathbf{V(fibnuc)}$, was calculated as:

$$\bar{v}_V(\text{fibnuc}) := \frac{\pi}{3} \cdot \overline{l_0^3} \tag{18}$$

where l_0^3 is the average cubed intercepts. On average, 124 intercepts were measured per left ventricle with a mean CE[$\bar{v}_V$(fibnuc)] of 0.12.

The number-weighted mean volume of fiber nuclei, $\bar{v}_N$**(fibnuc)**, was estimated from:

$$\bar{v}_N(\text{fibnuc}) := \frac{V_V(\text{fibnuc/LV})}{N_V(\text{fibnuc/LV})} \tag{19}$$

The coefficient of variation of volume in the number distribution, $CV_N(v)$, was obtained from:

$$CV_N(v) := \left[\frac{\bar{v}_V(\text{fibnuc})}{\bar{v}_N(\text{fibnuc})} - 1 \right]^{(1/2)} \tag{20}$$

(8) Estimation of the total length, volume, and surface area of fibers

The IUR thin sections were used. A 60X oil objective lens was used when measuring. Using a final magnification of 1400X, fields of vision were systematically randomly sampled in the sections using a fixed x- and y-step length (1200μm and 1200μm, respectively). The total length, volume, and surface area of the fibers in the left ventricle were estimated in the same way as the estimates of the total length, volume, and surface area of the capillaries in the left ventricle. The area of the counting frame was 3328 μm^2 at the tissue level. On the average, 210 fiber profiles were counted per left ventricle with a mean CE[L(fibers, LV)] of 0.19. On the average, 240 points hitting fibers were counted per left ventricle with a mean CE[V(fibers, LV)] of 0.06. On the average, 550 intersections were counted per left ventricle with a mean CE [S(fibers,LV)] of 0.15.

The mean cross-sectional area of fibers, $\bar{a}$**(fiber)**, was obtained from the following equation:

$$\bar{a}(\text{fiber}) := \frac{V(\text{fibers,LV})}{L(\text{fibers,LV})} \tag{21}$$

The mean volume of fiber per nucleus, V_N**(fib/nuc)**, was obtained according to the following equation:

$$V_N(\text{fib/nuc}) := \frac{V(\text{fibers,LV})}{N(\text{fibnuc,LV})} \tag{22}$$

(9) Estimation of the star volume of muscle fibers

The IUR thin sections were used to estimate the star volume of fibers. The star volume of any structure is defined as the average volume of the structure that is directly and

unobscured visible from any point inside the structure; the average is over all interior points of the structure. A 60X oil objective lens was used when measuring. Using a final magnification of 1400X, fields of vision were chosen systematically randomly with a step size of 1400 × 1400 μm^2. An integral test system, with points and lines, was superimposed on the sampled images. The star volume of fibers was obtained by the use of point-sampled intercepts (Gundersen and Jensen, 1985): for each point hitting the profile of a fiber in focus, the intercept length of fiber was measured from one border of fiber to the other border. The intercept lengths were individually cubed. The difference between star volume and mean volume-weighted volume is that star volume is estimated from the measurements of uninterrupted intercepts and mean volume-weighted volume is estimated from the measurements of all intercepts. Therefore, for the convex structure, two values are the same, but, for the concave structure, two values are different. The mean star volume of fibers, $v^*(\mathbf{fiber})$, was calculated as:

$$\overline{v^*}(\mathbf{fiber}) := \frac{\pi}{3} \cdot \overline{l_0^{*3}} \qquad (23)$$

where l_0^{*3} is the average, cubed, and uninterrupted intercepts of fibers. On average, 272 intercepts were measured per left ventricle with a mean CE[$\overline{v}^*$(fiber)] of 0.16.

(10) Estimation of the nuclear-volume-weighted star volume of muscle fibers

On the IUR sections used above, a new combination of stereological estimators was used to estimate the nuclear-volume-weighted star volume of fibers. A 60X oil objective lens was used and the final magnification was 1400X. The fields of vision were systematically randomly sampled in the sections with a step size of 800 × 800 μm^2. An integral test system, with points and lines, was superimposed on the sampled images. A fiber was sampled if a fiber nucleus was hit by a point. The two-uninterrupted distances from the sampling point to the boundary of the fiber profile were both measured along the 3D isotropic line, i.e., the nucleator principle (Gundersen, 1988) was used for size estimation. The mean nuclear-volume-weighted star volumes of fibers, $v^*_{\mathbf{V(nuc)}}(\mathbf{fiber})$, were calculated as:

$$\overline{v}^*_{\mathbf{V(nuc)}}(\mathbf{fiber}) := \frac{4\pi}{3} \cdot \overline{l_n^{*3}} \qquad (24)$$

where l_n^{*3} is the average, cubed, and uninterrupted distances from the sampling point to the boundary of the fiber profile. On average, 185 intercepts were measured per left ventricle with a mean CE[$\overline{v}^*_{V(nuc)}$(fiber)] of 0.15.

STATISTICS

Variability within groups was expressed using the dimensionless coefficient of variation. The observed overall variation of the estimates among individuals depends on the amount of sampling error at the different sampling levels and also on the inherent biological variation,

CV_{bio}, among individuals. The biological coefficient of variation among hearts was estimated from the calculated stereological sampling variation (the mean of the observed coefficient of error of the individual estimates, OCE) and the observed inter-heart coefficient of variation (OCV), which is calculated as SD/mean, using the relationship: $OCV^2 = CV_{bio}^2 + OCE^2$. The OCE was estimated from the estimated intra-left ventricle coefficient of error using the relationship: $OCE=\sqrt{\text{mean } CE^2}$. The CE of the Cavalieri estimator of the left ventricular volume, CE[V(LV)], in each heart was calculated in the following way.

The expected variance of point counting with a lattice of systematic points on n_b slabs, referred here to as the independent noise, **Noise**, was calculated according to the formula:

$$\mathbf{Noise} := 0.0724 \cdot (b/\sqrt{a}) \cdot \sqrt{n_b \cdot \Sigma P} \tag{25}$$

Where the value 0.0724 is a constant for point lattices with a quadratic arrangement, n_b is the number of slabs used, ΣP is the total number of points counted, and $b/\sqrt{a}$ is an average shape factor for a set of sectional profiles, i.e. the length of the profile boundary, b, divided by the square root of the profile area, $\sqrt{a}$. In this study, the shape factor 12 was used, which was obtained by comparing the overall shape of the sectional profiles with those presented in the nomogram that predicts the number of points necessary to perform counts (Gundersen and Jensen, 1987).

The variance of areas among the systematic random sample of slabs used to estimate the volume of left ventricle, **Var[a(LV)]**, was computed with the formula (Gundersen et al, 1998):

$$\mathbf{Var[a(LV)]} := [3(A - Noise) - 4B + C]/240 \tag{26}$$

where $A = \Sigma(P_i \cdot P_i)$, $B = \Sigma(P_i \cdot P_{i+1})$ and $C = \Sigma(P_i \cdot P_{i+2})$.

The total intra-left ventricle coefficient of error for the Cavalieri estimate of the left ventricular volume, **CE[V(LV)]**, was estimated as:

$$\mathbf{CE[V(LV)]} := \frac{\sqrt{Noise + Var[a(LV)]}}{\Sigma P_i} \tag{27}$$

P_i was the number of points hitting left ventricle on the *i*th coronal section.

The length, volume, surface area and numerical densities of the capillaries, the volume densities of fibers and fiber nuclei, the numerical density of the fiber nuclei, are ratio estimators, therefore, the equation in Kroustrup and Gundersen (1983), was used to estimate their CE in each heart according to the following formula:

$$CE_n\left(\frac{\Sigma Y}{\Sigma X}\right) := \sqrt{\frac{n}{n-1}\left(\frac{\Sigma(X)^2}{\Sigma X \Sigma X} + \frac{\Sigma(Y)^2}{\Sigma Y \Sigma Y} - \frac{2\Sigma(XY)}{\Sigma X \Sigma Y}\right)} \tag{28}$$

Where ΣX is the number of fields of visions used in each block when estimating the density parameter, ΣY is the object hit count in each block and n is the number of blocks used. The formula (Gundersen et al., 1993) which can be used to estimate the minimum coefficient error of the estimate of the capillary numerical density is:

$$CE := \frac{\sqrt{\#Bridges + \#Islands + \#Holes}}{\#Bridges - \#Islands - \#Holes} \qquad (29)$$

The estimated CE for the volume estimation of the left ventricle and the estimated CE for the density estimates of the capillaries, fibers, fiber nuclei and connective tissue nuclei were added together in the usual way to obtain the total CE.

RESULTS

As an illustration of the set of methods described the observations of the myocardial capillaries from six normal human left ventricles are presented in Table 1. The average volume of the normal left ventricle was 136 cm^3 with an observed CV of 0.15 and a CE of 0.04, i.e. the estimated biological variation is $\sqrt{0.15^2 - 0.04^2} = 0.14$. As expected, most total quantities varied somewhat more (range 0.16 to 0.36) with the myocardial fiber nuclear quantities in the high range of variation. The total number of capillaries in the left ventricle was $1.54 \cdot 10^9$ with a CV of 0.20 and the mean length of a capillary was 125 μm with a CV of 0.14. The number weighted mean volume of fiber nucleus was 62 μm^3 with a CV of 0.39, whereas the volume weighted mean volume of fiber nucleus was 259 μm^3 with a CV of 0.05. The $CV_N[\bar{v}(fibnuc]$ was 1.88. The various CE's are given under the description of the estimators.

With respect to design efficiency, it may be noted, that for most parameter estimates the biological variance constitutes most of the total observed variance, from 54% to 93%, i.e. the added variance from the sampling and estimation procedure was a minor component.

DISCUSSION

The number of blocks, the number of fields of vision, and the intensities of the test system used in each left ventricle for the estimates of different parameters constitute the overall sampling scheme. The sampling scheme used in this study was selected on the basis of the general principles presented in the method section and on data from a pilot study. For example, two different intensities of test points were used to estimate the fiber density and fiber nucleus density because of the large difference between fiber fraction and fiber nucleus fraction. When measuring the volume-weighted mean volume of fiber nuclei and the nuclear-volume-weighted star volume of fibers, we sampled more fields of vision with a small step size and used denser test points because the volume fraction of nuclei is very small. When estimating the number of fiber and connective tissue nuclei, we used two different sizes of unbiased counting frames because of the large difference of the numerical densities for these two cell populations. The sampling schemes were designed to obtain a hit count or a number of measurements of 100 to

200 events or hits for a complete ventricle. As mentioned in the method section, the evaluation of the precision of the sampling scheme was based on the estimates of the observed total variance (OCV^2) and the stereological sampling variation (OCE^2). In other words, whether or not the precision of the sampling scheme is sufficient depends on the biological variation, which was unknown until the analysis of the six human left ventricles had been performed. The biological variation is easily estimated by subtracting the estimated stereological sampling variance of the individual estimate of total quantities of fibers and capillaries (OCE^2) from the total observed variance (OCV^2) (Kroustrup and Gundersen, 1983). Much of the total variance observed may be attributed to variation among the left ventricles, i.e., to the biological variation, but evidently the estimates of biological variation is preliminary due to the fact that the sample of 6 hearts is small and the material is not intended to be particularly homogeneous. As an illustration of the hazards of small materials for the estimation of variability the $CV\left[_{VV}(fibnuc)\right]$ of 0.05 is an unrealistically low, which is less than a half of the expected CE. The primary reason for including all the estimates of variability is that we feel it pertinent to underline that and illustrate how such estimates are made and for which purpose they are used.

For most of the measurements, the precision of the individual stereological estimates is satisfactory in the sense that the major factor contributing to the total observed inter-individual variability was the fixed, inherent, biological variability of the individuals of the sample studied and not the precision of the individual stereological estimates. Considering the small contribution to the overall variance caused by the stereological sampling for the most of the measurements, this is clearly too precise, and it would be more efficient to decrease the measurements using a test system with coarser test points and test lines. This is especially the case when estimating the capillary surface area where the number of intersection counting should be decreased by 50% using every second test line. For the estimates of the total capillary length, the total fiber length, and the volume-weighted mean volume of fiber nucleus, the stereological sampling variation contribute most to the total observed variance. For these estimates, the sampling variances at the second level, individual block, are analyzed in the same way as above mentioned and the stereological sampling variation among blocks for the estimation of the capillary length, the fiber length, and the volume-weighted mean volume of fiber nucleus contribute 24%, 17%, and 14%, respectively, to the observed inter-block variance. Therefore, for these estimations the number of blocks, i.e. 6 blocks in this study, rather than the number of measurements in each block need to be increased if and only if the biological variation is at the level observed in the small sample of 6 human hearts.

CONCLUSION

Based on unbiased principles, this study presents new design-based stereological methods to obtain scientifically reliable estimates of the total amounts and sizes of capillaries, fibers, fiber nuclei and connective tissue nuclei in the human left ventricle. The combined impact of the stereological methods is that all total quantity estimates are independent of any tissue inhomogeneity. All estimates of average size are correct for the complete left ventricle and require for their interpretation only that inhomogeneities do not leave a global average questionable. The estimates were carried out using light microscopy and plastic sections

presumably without any noticeable problem with tissue deformation. The presented methods may not only be applied to any region or part of heart in developmental, comparative, pathological and experimental studies, but may also be used to make quantitative investigations of muscle fibers and capillaries of any separate region or complete organ in different research projects.

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Table 1: The left ventricular data and capillary quantities, left ventricular volume, V(LV), volume fraction of capillaries, V_V(cap), total volume of capillaries, V(cap), numerical density of capillaries, W_V(cap), total number of capillaries, W(cap), length density of capillaries, L_V(cap), total length of capillaries, L(cap), surface area density of capillaries, S_V(cap), total surface area of capillaries, S(cap), and coefficient of error of the individual estimates, CE.

	Sex	V(LV)	CE V(LV)	V_V(cap)	V(cap)	CE V(cap)	W_V(cap)	W(cap)	CE W(cap)	L_V(cap)	L(cap)	CE L(cap)	S_V(cap)	S(cap)	CE S(cap)
		cm^3		10^{-1}	cm^3		$10^2\ mm^{-3}$	10^9		mm^{-2}	km		mm^{-1}	m^2	
1	female	118	0.03	0.36	4.25	0.13	112	1.33	0.17	1276	152	0.09	26.0	3.09	0.12
2	female	119	0.06	0.25	2.98	0.10	101	1.20	0.11	1318	157	0.09	17.4	2.08	0.08
3	female	129	0.04	0.35	4.52	0.11	101	1.30	0.07	1586	205	0.12	22.4	2.89	0.07
4	female	129	0.04	0.45	5.81	0.06	135	1.74	0.07	1597	206	0.18	25.2	3.25	0.05
5	male	155	0.03	0.40	6.20	0.11	105	1.62	0.08	1260	196	0.13	27.3	4.24	0.09
6	male	165	0.05	0.35	5.78	0.12	122	2.02	0.09	1303	216	0.16	23.1	3.82	0.09
Mean		136	0.04	0.36	4.92	0.11	113	1.54	0.10	1390	189	0.13	23.6	3.23	0.09
SD		19.6		0.07	1.23		13.5	0.31		157	27		3.53	0.75	
OCV		0.15		0.19	0.25		0.12	0.20		0.11	0.14		0.15	0.23	
$1-CE^2/OCV^2$		0.93			0.81			0.75			0.14			0.85	