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Quantitative morphology of the vascularisation of organs: A stereological approach illustrated using the cardiac circulation

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SUMMARY

The vasculature of the heart is able to adapt to various physiological and pathological stimuli and its failure to do so is well-reflected by the great impact of ischaemic heart disease on personal morbidity and mortality and on the health care systems of industrial countries. Studies on physiological or genetic interventions as well as therapeutic angiogenesis rely on quantitative data to characterize the effects in a statistically robust way. The gold standard for obtaining quantitative morphological data is design-based stereology which allows the estimation of volume, surface area, length and number of blood vessels as well as their thickness, diameter or wall composition. Unfortunately, the use of stereological methods for this purpose is still rare. One of the reasons for this is the fact that the transfer of the theoretical foundations into laboratory practice requires a remarkable amount of considerations before touching the first piece of tissue. These considerations, however, are often based on already acquired experience and are usually not dealt with in stereological review articles. The present article therefore delineates the procedures for estimating the most important characteristics of the cardiac vasculature and highlights potential problems and their practical solutions. Worked examples are used to illustrate the methods and provide examples of the calculations. Hopefully, the considerations and examples contained herein will provide researchers in this field with the necessary equipment to add stereological methods to their study designs.

1. Introduction: Cardiac plasticity

Despite the extremely limited capacity of the heart to replace lost cardiomyocytes (Laflamme and Murry, 2011), there is a wide range of physiological and pathological conditions to which the heart can adapt by functional and/or structural changes (Hill and Olson, 2008). The ability of the heart to adapt to systemic needs by hypertrophic or atrophic remodelling is well illustrated by its responses during exercise (Mattfeldt et al., 1986; Eisele et al., 2008), increased pulmonary or systemic arterial pressure (Anversa et al., 1989), unloading (Brinks et al., 2009), changes in body weight (Gruber et al., 2012a,b), postnatal growth (Anversa et al., 1980; Olivetti et al., 1980) and pregnancy (Bassien-Capsa et al., 2006; Fidzianska et al., 2010). Structural remodelling of the heart involves changes in cardiomyocyte size, number, ultrastructure and intercellular contacts as well as alterations of the microcirculation, extracellular matrix, composition of interstitital cells and innervation. Many changes can be quantified by stereological estimation of various parameters, such as number, length, surface area or volume (Mühlfeld et al., 2010a; Tang et al., 2009; Weibel, 1979, 1980). During the past few years, estimating the number of cardiomyocytes (Brüel and Nyengaard, 2005) and the total length of axons (Mühlfeld et al., 2010b) have complemented the spectrum of methods for analyzing heart morphology. Table 1 provides some examples of design-based stereological studies on the vasculature of the heart. As can be seen from this table, some information is available for human, mouse and rat hearts under normal and pathological conditions but mostly the left ventricle has been investigated alone. There is only limited information on the right ventricle, on other stereological parameters such as the composition of the vessel walls and on parts of the vascular system apart from capillaries.

The most common morphometric approach for analyzing remodelling of the cardiac microcirculation is to count the number of vessel profiles per unit sectional area. Unfortunately, as outlined below, this may be accompanied by various sources of bias and misinterpretation. Therefore, the present review article gives an overview of the potential pitfalls in cardiac blood vessel quantification and provides practical details of how these problems can be overcome.

2. Morphometric parameters related to vasculature

Viewed from an anatomical–physiological perspective, each of the four global parameters (number, length, surface area and

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Table 1
A few examples of studies using design-based stereology in the analysis of the vasculature of the heart.

Study	Species, sex, organ	Part of vascular system	Data	Observed change under experimental condition
Tang et al. (2009)	Human ($n=6$), 4 × female/2 × male, left ventricle	Capillaries	$V = 136\ cm^3$ (SD 19.6) $N = 1.54 \times 10^9$ (SD 0.31) $L = 189$ km (SD 27) $S = 3.23\ m^2$ (SD 0.75)	–
Gruber et al., 2012a	Mouse ($n=5$), male, left ventricle	Capillaries	$V = 3.65\ mm^3$ (SD 1.47) $L = 450$ m (SD 94)	Decrease due to caloric restriction
Gruber et al., 2012b	Mouse ($n=5$), male, left ventricle	Capillaries	$L = 340$ m (SD 52)	Increase due to obesity
Wiest et al., 1992	Rat ($n=10$), male, left ventricle	Arteries	$L = 2.48$ m (SD 0.33)	Increase during ageing
Brüel et al., 2005	Rat ($n=3$), female, left ventricle	Capillaries	$V \approx 0.024\ cm^3$ $L \approx 0.9$ km	Increase due to treatment with growth hormone
Warley et al., 1995	Rat ($n=8$), male, left ventricle	Capillaries	$L = 2.93$ km (SEM 2.03) $S = 503\ cm^2$ (SEM 26)	Decreased surface area due to diabetes
Amann et al., 2002	Rat ($n=7$), male, left ventricle	Capillaries	$L = 3.36$ km (SD 0.97)	Decrease due to partial renal ablation was reduced by antioxidant therapy

V = volume, N = number, L = length, S = surface area, SD = standard deviation, SEM = standard error of the mean.

volume) may provide meaningful information on the vascular bed of the heart. In addition, stereology provides tools for estimating the mean diameter of blood vessels and the mean thickness of the vessel wall. However, attention must first be paid to identification of those parameters which are most useful in a particular study design.

Generally, a distinction between different parts of the circulation (i.e. between arteries, arterioles, capillaries, venules and veins) is useful although difficulties will arise when trying to distinguish between arterial and venous blood vessels solely based on morphological criteria. Antibody staining has been employed by various authors to stain the arterial and venous compartments differentially (Lojda, 1979; Batra and Rakusan, 1991a,b; Koyama et al., 1998).

2.1. Volume

Volume estimation is a straightforward and simple way to gain information on the overall content of blood vessels in the heart. Initially, it has to be decided whether volume estimation should include both the lumen and the vascular wall. Vascular luminal volume is proportional to the blood volume contained within the myocardial circulation. Therefore, it is useful to estimate the volume of the vascular lumen separately from the volume of the wall. Of course, account must be taken of the fact that the luminal volume depends on the mode of preparation and fixation. Physiological perfusion of the myocardial circulation shows significant spatial and temporal variations (Schwanke et al., 2000). In addition, the normal contraction and relaxation cycle influences the perfusion status of blood vessels (Boudoulas et al., 1979). It is therefore advisable to fix all hearts in a similar contraction status, e.g. by arresting the heart in diastole. This can be achieved by perfusing the heart with a hyperkalaemic solution similar to the ones used for cardioplegic arrest in heart surgery (Schmiedl et al., 1993). Perfusion fixation at a sufficient pressure is the gold standard for heart fixation at least if transmission electron microscopy (TEM) is used (Hayat, 2000). However, a thorough perfusion will open all blood vessels to a similar degree, thus neglecting the regional differences of cardiac blood supply. In a perfusion-fixed heart, most blood vessels (including small capillaries) will be visible at the light microscopic level and this makes it possible to perform stereological analyses without resorting to electron microscopy.

Fixation of the whole heart by immersion provides good morphological results (Eisele et al., 2008) but is only possible for small hearts such as those of mice. Larger hearts will require cutting small pieces for immersion in the fixative which affects the morphology of the myocardium to a varying extent (Schaper et al., 1985). When animals are killed by exsanguination under anaesthesia, the blood volume within myocardial vessels will most likely be affected although no systematic studies are available. As the immersion of the hearts results in an intermediate contraction status and the blood vessels are either filled with blood cells or are collapsed, it is hardly possible to identify all small capillaries. In this case, for a reliable estimation of vessel volume, TEM has to be applied (Gruber et al., 2012a,b). For the estimation of vascular wall volume TEM is recommended in any case as the small volume of the capillary endothelium cannot be estimated with enough accuracy by light microscopy. The thickness of the vascular wall may be of interest when changes in its composition (e.g. an increase in smooth muscle cells or extracellular matrix) are thought to occur (Amann et al., 1995) or when the diffusion distance from capillary lumen to cardiomyocytes is being investigated (Warley et al., 1995). While the first issue may require estimating the volumes of different components of the vascular wall, the diffusion distance can be estimated as the arithmetic or harmonic mean thickness of the wall (see below).

2.2. Surface area

The endothelial (luminal) surface area of capillaries is a useful parameter because it offers a measure of the maximum surface area for diffusion of oxygen and nutrients to the cardiomyocytes. In combination with the total blood volume contained in the myocardial capillaries, it provides useful information on the supply of cardiomyocytes. Capillary endothelial surface area may also be related to cardiomyocyte surface area. Changes in this ratio, e.g. in hypertrophy, may indicate important changes in the supply status of the cardiomyocytes.

Basically, the considerations on heart fixation addressed above also relate to surface area estimations. In addition, surface area estimations are extremely sensitive to the microscopic resolution. This can be illustrated by the famous “Coast of Britain” example (Mandelbrot, 1967): seen from a height of a thousand miles, the coast of Great Britain presents as a more or less smooth boundary. The closer we get, the more details (such as bays, coves, peninsulas or tongues of land) will become visible and the estimation of the surface area will therefore increase the smaller the distance gets. Thus, a good estimate of endothelial surface area should be based on TEM images.

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