# Scanning Electron Microscopy and 3D Morphometry of Vascular Corrosion Casts: Techniques and Current Applications in Biomedical Research

*Minnich B[1]; Sick C.[2]*

**Abstract**

**Background**: Vascular corrosion casting is an old method that has found application by anatomists for a long time. Initially, the viscosity of casting media was very high and thus the technique failed to cast the entire circulatory system from the arterial injection site through small arteries, arterioles, capillaries, venules, small veins and caval veins. Casting media soon were improved and thus enabled casting of the entire circulatory system. Examination of these vascular casts under the light microscope does not allow a thorough analysis with high resolution. Only when in 1971 Murakami applied Scanning Electron Microscopy SEM. to study vascular corrosion casts a breakthrough was achieved and for the first time, microvascular networks, too, were able to be analysed with a spatial resolution high enough to study the surface of the casts also termed injection replicas. and a depth of focus high enough to examine a large field of view enabling to reliably trace individual vessels either over very short or over extremely long distances.

Scanning electron microscopy SEM. is a tool with great potential for morphological research. The images generated via SEM have great resolution and a high depth of focus, which gives SEM micrographs pseudo three-dimensionality. Adversely, the high depth of focus prevents accurate dimensional or spatial measurements of imaged microstructures from either the SEM video-display, printed micrographs or from photonegatives. Macroscopic objects are viewed close up using binocular vision. Binocular vision is also used in microscopy where stereophotogrammetry and related techniques applying stereo paired images, and a variety of hardware tools calculate the third dimension z-coordinate. using the parallax.

Morphometric SEM 3D analysis is currently used to analyse i. the geometry of microvascular trees in terms of vascular parameters i.e., diameters, interbranching distances, branching angles and intervascular distances. and ii. to determine bifurcation indices, asymmetry and area ratios given in arterial bifurcations respectively in venous mergings. Moreover, it allows to generate anaglyphic 3D images and to calculate optimality principles underlying the construction and maintenance of such delicate vascular networks i.e. principles of minimal lumen volume, minimal pumping power, minimal lumen surface and minimal endothelial shear force.

**Aim:** The main goal of the method is to characterize microvascular networks in human and animal tissues in order to demonstrate their anatomical situations and to reflect onto their physiological conditions. These methods enable not only to qualitatively describe but also to quantitatively explore the microvascularisation by means of three-dimensional morphometry and thus to compare different developmental stages and progress of disease.

**Conclusions:** The combination of the techniques described above allows a thorough study of microvascular networks in healthy- and pathologic tissues and organs. This enables i. to enhance the knowledge of vascular- / anatomical situations and development, ii. to compare the physiological conditions in health and different pathologies and iii. to gain insights and to develop therapeutical strategies in various clinical pictures, which are critically dependent on a proper functioning vascularization **(TCM-GMJ December 2023; 8 (2):P14-P19)**

**Keywords:** Vascular Corrosion Casting VCC.; Scanning Electron Microscopy SEM.; 3D-Morphometry; Bio-/ Medical Applications of VCC;

## Introduction

The cardiovascular system of animals and humans is the first system to develop and to function. Failure of the system results in severe functional disruption and, in more severe cases, premature death. At present, failure of this system, also known as cardiovascular death, has become the leading cause of death in Western countries (1).

Arteries, capillaries, and veins are all arranged in a hierarchical order forming arterial, capillary, and venous trees. In most tissues and organs vascular trees have been shown to display a three-dimensional arrangement, in a few cases only they are flat and form two-dimensional structures. In general, arterial trees gradually change into capillary trees. Postcapillary venules in turn merge to form venous trees. In special situations, however, arterial trees directly interconnect via arterio-arterial anastomoses with other arterial trees or interconnect with venous trees via arterio-venous anastomoses. Lastly, veno-venous anastomoses interconnect venous trees.

Arterial trees form by repeated bifurcations of a parent (stem, mother) vessel of diameter $d_0$ into two daughter vessels with diameters $d_1$ (thicker daughter vessel) and $d_2$ (thinner daughter vessel). These three diameters together with the branching angles between the parent vessel and the thicker branch ($\theta_1$) and the parent vessel and the thinner branch ($\theta_2$) then characterize the simplest vascular tree (2-4). By taking then

*From the [1] Paris-Lodron University of Salzburg, Dept. Environment & Biodiversity, Vascular Biology Unit, Hellbrunnerstr. Salzburg, Austria ; [2]Kepler University Hospital, Department of Neurology, Salzburg, Austria;*
*Received October 3, 2023; accepted December 10 , 2023.*
*Address requests to: Bernd Minnich*
*E-mail: Bernd.Minnich@plus.ac.at*
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each of the two daughter branches as a new parent vessel and bifurcating each of these vessels after a certain distance (interbranching distance, ib) again and again, a vascular network forms whose geometry can be described exactly using the denomination system proposed by (2). In case of venous trees, two daughter vessels merge into a single, then thicker parent vessel (vein). There is, however, not yet sufficient evidence available (5), if venous mergings may be dealt with in the same manner as we presently do with arterial bifurcations.

From the many methods available to analyse blood vessels both in- and ex-vivo, our focus lies on scanning electron microscopy (SEM) of vascular corrosion casts/ injection replicas (6,7-9) combined with 3D-morphometry (10-12). We measure diameters, lengths, intervascular- and interbranching distances as well as angles in arterial bifurcations (mergings in venous trees) from stereo-paired SEM images using a 3D-morphometry system *M3* (*ComServ OG*, Ebenau, Austria). With these data we can test which of the four given optimality principle(s), i.e. (i) minimum pumping power (5), (ii) minimum resistance (13), (iii) minimum volume (14,15) and (iv) minimum drag force (16,17) underlies the design of selected arterial bifurcations.

The method for dimensional- and angular measurements of microstructures imaged in the SEM was first developed in our lab in 1999 (10). It uses digitized stereo paired images frame-grabbed (slow scan) directly from the SEM's photo-display, vector equation-based algorithms for the calculation of spatial coordinates and derived distance as well as angular measurements. It offers dynamic data exchange to MS Excel™ spreadsheets together with online graphs of frequency distributions of measured variables. Formulas for central perspective depth computation allow the overall error to be less than 1.0%. Meanwhile, the method was further improved and the new java-based software *M3* is suitable to be used with Windows 7, 10 or 11, Linux and Apple operating systems as well. A modern graphical user interface (GUI) and a new open-source data interface (Apache OpenOffice™) is supported, which facilitate the measurement processes. With the availability of this PC-based 3D-morphometry system previous qualitative analyses of casted vascular beds can be quantified and data of vessel (luminal) diameters, intervascular- and interbranching distances as well as of branching angles can be acquired. This enables to test existing microvascular networks with respect to given optimality principles underlying the construction and maintenance of individual vascular beds of tissues and organs in health and disease.

## Methods

Vascular Corrosion Casting (VCC)

About 15 minutes prior to the anaesthesia of the animal and injection of the rinsing respectively casting medium, heparin (1000 I.U./ml) should be applied systemically (s.c. or i.m.) to avoid blood coagulation within the fine capillaries preventing complete filling of the vascular bed. Depending on the target for microvascular corrosion casting, the injection site will be either the animal's heart (in the case of mammals the left ventricle) with ascending aorta, the descending aorta, or the caudal vena cava (orthogradely). In case of whole-body vascular castings, the heart is exposed, and the resin is administered via the ventricle (common or left). In the case of casting the vascular bed of a selected organ the injection site will be the artery which supplies the whole organ. This is true for the organ in-situ as well for the isolated organ. For practical work it is most important to select an injection site, which enables the particular vessel to be cannulated with sufficient certainty, ligatures to be put around vessels to fix the cannula or tubing, and which can be viewed clearly and illuminated not only from the top but also from the side. For practical work the rule is to inject as close to the target as possible (18).

According to Poiseuille's law, blood flow (as the important parameter for freeing the blood vascular system from blood cells and for transport of polymerizing casting media) decreases with increasing vessel length, i.e. the distance between injection site and vascular bed to be cast. Surgical care has also to be given to close as many large "through-ways" as possible, may they be large cut arteries or veins, by appropriate ligatures to prevent the injected polymerizing material from a too early escape through large open vessels (18).

Cannulation of the arterial feeder is done either with a micromanipulator or by hand depending on the calibre of the artery. To enable a clear view at the site where the cannula or the tubing can be introduced into the arterial feeder, we use a stereomicroscope with a 40x magnification. This has the limitation that the depth of focus is very low and the tip of the cannula, which must be guided with a rather oblique angle towards the arterial feeder will be in focus only over a very limited range. As cut arterial vessels tend to occlude their lumen by contracting their wall towards the lumen only a cannula with a tip-diameter much smaller than the diameter of the arterial feeder will glide easily into the vessel and allow to be pushed forward inside the vessel to finally be securely tied in place by the ligature (10-0 or 11-0 suture material). On the other hand, the smaller the lumen of the glass cannula, butterfly-needle or tubing is, the higher is the pressure needed to inject the casting medium and the greater is the risk that the polymerizing resin with its increasing viscosity cannot be injected for a sufficient time period to cast larger areas of the vascular bed. Ideally, the lumen of the cannula should be as large as possible for any given outer diameter of a cannula, which in turn must be seen in relation to the size of the lumen of the blood vessel it has to be introduced into. When the cannula is securely ligatured in the arterial feeder, rinsing with 0.9% saline is started. A first indicator for correct cannulation of an arterial feeder is blood efflux from the closest venous drainer as well as the wall of the artery turning a whitish colour. A retrograde injection via the much larger venous drainer does not work. Injected resin will stop distribution very soon or – if higher pressure is applied – cause rupture of the vessel. The injection of the liquid polymerizing resin (Mercox-Cl-2B, Ladd Re-

search Inc., Burlington, Vermont, USA), diluted with monomeric methylmethacrylate (4+1, v+v, 10 ml monomeric methylmethacrylate contain 0.85g initiator paste MA) is done by a perfusor (syringe) pump with a flow rate of 3 - 100 ml/hour depending on the tissue / organ / animal to be cast. A glance through the stereomicroscope at the injection site immediately informs whether the cannula is tightly secured in the arterial feeder. Consequently, there is no leakage of blue resin. After a few seconds however, resin reflux will start to escape the cut open sites. Injection is stopped when the effluent resin becomes highly viscous (19). During injection it is essential to avoid any air bubbles to be introduced into the vasculature as this would lead to vessel occlusion and subsequently the casting to fail.

## Preparation for Scanning Electron Microscopy (SEM) and SEM Inspection

### *SEM preparation*

Animals are left untouched for at least 30 minutes at room temperature (20°C) on the wax-plate to allow polymerization of the injected resin. Injected animals are then de-pinned carefully, placed into a water bath (60°C, 12 hours) for tempering, and finally macerated in KOH (7.5%, 40°C, 12 - 48 hours). When maceration is completed, vascular casts are transferred using a small spoon or a small vial always covered by the proper fluid via several passages of distilled water into formic acid HCOOH (5%, 20°C, 5-10 minutes), rinsed again in several passages of distilled water, frozen in distilled water and freeze-dried. Dry specimens are mounted onto specimen stubs using the "conductive bridge method" (20) and sputtered with a thin layer of gold. With this technique "conductive bridges" are fixed under stereomicroscopic control to individual vessels with colloidal silver paste. Thereby, viscous colloidal silver must be used because too fluid colloidal silver will be sucked up into capillary meshes masking individual vessels. Furthermore, interesting details of the specimens should not be hidden by the attached "conductive bridges" (19).

### *SEM inspection*

Coated specimens are investigated under a scanning electron microscope (Philips ESEM XL-30, FEI, Eindhoven, NL) at an accelerating voltage of 5-10 kV. This accelerating voltage is high enough to investigate all those details at the (luminal) surface of casted vessels which give useful information (e.g. cell nuclei imprints). Accelerating voltages higher than 10 kV will cause a higher thermal burden to the specimen with the risk of damaging the specimen. The higher spatial resolution gained when the SEM is operated with higher accelerating voltage it will display the limits of the replication quality of the injection resins and it will reveal even the proper structure of the resin used if for example a high-resolution scanning electron microscope (HRSEM) is used to inspect vascular corrosion casts (19). For consecutive 3D morphometry stereo-paired images (tilt angle: 5-12°; working distance: 10 – 30 mm; resolution: 480 x 480 px) are taken and transferred into the *M3* analysis software (21).

## Three-dimensional (3D) Morphometry

Digital stereo paired images are imported into the 3D-morphometry analysis system *M3* (*ComServ, OG*, Ebenau, Austria). To gain spatial orientation in the SEM-images, 3D representations are generated based on the anaglyphic red/green method by super-positioning (averaging) the previously complementary coloured stereopairs. Morphometric measurements performed on the original 480 x 480, 8 bits grey level images consider the depth of focus and thus the third dimension in the SEM by calculating the parallax. Therefore, the type of projection (i.e. parallel projection (magnification (mag) > 500 x and working distance (wd) > 10 mm) respectively central perspective projection (mag $\leq$ 500x and wd $\leq$ 10 mm)) and the field of view (fw) are critical parameters when acquiring the stereo-paired images (e.g. tilt = 6 ± 0.1°). Every point in 3D (x,y,z) is computed from its corresponding 2D-plane co-ordinates (s,t) and (u,v) obtained by point-setting using the computer's input device. For mathematical reasons, we first scale the planar stereopair co-ordinates onto a (-1,1) interval within a cartesian system of co-ordinates and then use homogeneous co-ordinates (x,y,z,1) for computing the neces-

$$x = \frac{10}{N} \cdot (4\, s\, v\, (\sin(\gamma)\, \sin(\rho/2))^2 + s\, \sin(2\gamma)\, \sin(\rho)), \quad (1)$$

$$y = \frac{10}{N} \cdot ((t + v)\, \sin(\gamma)\, \sin(\rho)), \quad (2)$$

$$z = \frac{10}{N} \cdot (4\, t\, v\, \sin(\gamma)\, (\sin(\rho/2))^2 + (t - v)\, \cos(\gamma)\, \sin(\rho)), \quad (3)$$

whereby $N = \sin(2\gamma)\,(1 + \cos(\rho)) + t\, v\, \sin(2\gamma)\,(1 - \cos(\rho)) + (t - v)\, \cos(2\gamma)\, \sin(\rho)$.

For the parallel perspective projection, we obtain the well-known formulas:

$$x = \frac{\mu\, s}{2} \qquad y = \mu \cdot \frac{t + v}{4 \cos(\gamma)} \qquad z = \mu \cdot \frac{t - v}{4 \sin(\gamma)} \quad (4, 5, 6)$$

sary transformation matrices and finally apply the appropriate formula to calculate a point's spatial co-ordinates (10) Eq. (1-6). (6)

For a central-perspective projection denote the angle of its field width (fw) by r and the tilt angle by r and angle by g.

A tedious but straight-forward computation gives:

Once 3D-points are calculated, simple trigonometric vector equations are used to calculate (i) a distance (l) between two points, respectively (ii) an angle (θ) between three points in 3D space. The resulting measures are then comfortably exported into OpenOffice spreadsheets and frequency distributions are displayed simultaneously therein. The accuracy of this method was proofed to be 1 ± 0.5% (10), a comparison between 2D and 3D measurements resulted in up to 60% underestimation of lengths measurements due to the missing spatial information in 2D (6,8).

Calculation of Vascular Optimalities

3D geometry data are then used to test which of the four given optimality principle(s), i.e. (i) minimum pumping power (5,22), (ii) minimum resistance (13), (iii) minimum volume (15,16), and (iv) minimum drag force (16,17) underlies the design of a certain (micro-) vascular network in terms of its construction and maintenance. For this we have implemented a so-called bifurcation function which enables to specify consecutive branchings within a symmetric- or an asymmetric vascular tree.

A bifurcation is characterized by the diameter $d_0$ of the parent artery, the diameters $d_1$ and $d_2$ of the two daughter branches and the branching angles $\theta_1$ and $\theta_2$ at which the two branches arise from the parent vessel. The convention is made, that $d_1$ always denotes the larger of the two branch diameters. A useful variable in the study of bifurcations is the so called "bifurcation index" α, which represents the ratio of the smaller branch diameter to the larger one. This index varies from 0 to 1. For a given value of the bifurcation index there are optimum values of the branch diameter ratios $d_1/d_0$ and $d_2/d_0$ (23). The use of actual diameters is associated with difficulties and errors, particularly if the measurements are taken from a magnified image of the vessels. These problems can be avoided by specifying a bifurcation in terms of relative rather than actual diameters. This is achieved by introducing two non-dimensional parameters, the area ratio b and the asymmetry ratio a` (21). The angles that the two branches make with the parent artery at an arterial bifurcation are such as to minimize the volume of blood that the bifurcation region must contain and, at the same time, minimize such fluid dynamic factors as the pumping power required to drive blood through the bifurcation and the shear force between moving blood and endothelial tissue. Although the first of these conditions conflicts with the second and third, there is for each branch an "optimum" branching angle that would produce a compromise (24). The angles the two branches span with the direction of the parent vessel have also been determined optimally to depend on the bifurcation index α (25).

The results of these calculations are then transferred into OpenOffice spreadsheets and scatter plots illustrate which of the four given optimality principles is best representing the vascular network under examination. This allows us to better understand which different strategies underly the construction and maintenance of vascular networks of tissues and organs during maturation, in health, disease and rehabilitation in different animal species and humans (21).

## Results and discussion

Vascular corrosion casting and described associated techniques not only allow a thorough insight into the (micro)vasculature of selected organs and tissues in health and disease but also enable to quantify the angioarchitecture by means of geometric measures. In turn this serves as the basis for exploring the anatomy, the physiology, and the optimality of blood vascular systems in terms of their construction and maintenance. Inspection of vascular casts under the scanning electron microscope also enables the:

*(i) Identification of cast vascular structures*

Due to the high replication quality of today`s casting media, casted arterial vessels can be reliably differentiated from venous ones by (i) their characteristic endothelial cell (nuclei and border lines) imprint patterns and (ii) the "gross" appearance these two vessel types reveal in terms of vascular profiles (round, ovoid, flat), branching patterns (bifurcations, trifurcations), and interbranching distances. Endothelial cell nuclei imprint pattern found at the surfaces of cast arterial vessels are oval to longish and are oriented parallel to the vessel long axis. Those of venous vessels are roundish to oval and show no specific orientation in respect to the long axis of the vessel. Capillaries are identified by their small diameter. Sinusoids which have a larger diameter than capillaries can be identified by their positions between precapillary arterioles and postcapillary venules, and between terminal portal venules and postcapillary hepatic venules (1).

*(ii) Identification of flow governing structures*

Beside distinction of basic vessel types a series of hemodynamically important structures can be replicated and identified in vascular casts. These structures encompass ve-

nous valves, flow dividers, intra-arterial (intimal) cushions and arterial and venous sphincters (1).

*(iii) Identification of artifacts*

Like any other technique, vascular corrosion casting is prone to artifacts. To enable correct conclusions from vascular corrosion casts, real existing structures must be differentiated from artifacts (26,27). Artifacts found in vascular corrosion casts are extravasations (evasates = extravasates), incomplete fillings, holes from air-bubbles or thrombi trapped by the casting medium, and plastic strips representing plasticized vascular smooth muscle cells (VSMCs) and/or pericytes (1,28).

# Conclusions

Recent Applications of Vascular Corrosion Casting in Medical Research

A current literature search listed the methodical involvement of the vascular corrosion casting technique in various medical fields, respectively diseases, namely (i) tumors, (ii) heart diseases, (iii) hypertension, (iv) diabetes, (v) lung diseases, (vi) kidney diseases, as well as (vii) liver fibrosis.

Here we briefly list some key publications and sum up their main study goals:

*(i) tumors:* Reference (29) focused on hepatic vasculature, interventional techniques and radiofrequency or microwave hyperthermia; diethylnitrosamine (DENA)-induced HCC in pigs for in situ HCC with a tumor latency of 10-26 months. Reference (30) focused on simulating pancreatic resections in different planes and find the optimal resection line with the minimum number of cut vessels.

*(ii) heart disease:* Reference (31) focused on the optimization of the vascular corrosion method to visualize the complete vascular tree of adult zebrafish to examine increased reproducibility and accuracy. Reference (32) focused on corrosion casting for an accurate diagnosis and space confirmation of congenital heart disease. Reference (33) used the modified vascular corrosion casting technique to prepare fetal cardiovascular casts with DA anomalies to analyse the great vessels of the fetal heart and to depict the fetal DA abnormalities from different fetal congenital heart defects (CHDs). Reference (34) focused on the preparation of fetal cardiovascular casts with the help of a modified vascular corrosion casting technique with fetal total anomalous pulmonary venous connection (TAPVC) for better demonstration and diagnosis of TAPVC. Reference (35) focused on the Inflammation and vascular remodeling in COVID-19 hearts. For the first time, they have observed intussusceptive angiogenesis in cardiac tissue, which they previously identified as the linchpin of vascular remodeling in COVID-19 pneumonia, as a pathognomic sign in affected hearts. *(iii) hypertension:* Reference (36) focused on the investigation of the three-dimensional structure of vascular lesions in the lung explant of a patient diagnosed with pulmonary veno-occlusive disease PVOD and PCH. Reference (37) focused on the impact of the changing vascular resistances on the hepatic and global circulation hemodynamics during cirrhogenesis. Peeters *et al.* (38) focused on the 3D architecture of the hepatic vasculature during induction of cirrhogenesis in a rat model. *(iv) diabetes:* Sandech *et al.* (39) focused on testing diabetic rats for the function of the vasoprotective gymnemic acid by examining both, microvessels in the brain and the release of the proteins VEGF and angiopoietin-1 (Ang-1). In reference (40) the microvascular morphology and pathological changes in gestational diabetes mellitus (GDM) placentas and normal placentas were observed. Vascular structure and histological morphology changes in GDM placentas were examined to generate basic experimental data for the diagnosis and prognostic determination of GDM. *(v) lung diseases:* In reference (41) the authors examined the associated morphologic- and molecular changes in the peripheral lung of patients who died from Covid-19. Reference (42) deals with the identification of specific variants of neoangiogenesis in three common pulmonary injury patterns, usual interstitial pneumonia (UIP), nonspecific interstitial pneumonia (NSIP) and alveolar fibroelastosis (AFE) in human interstitial lung diseases (ILDs). *(vi) kidney diseases:* Reference (43) evaluated the feasibility of using a novel biomimetic scaffold for kidney regeneration in a rat kidney cortical defect model. Zambon *et al.* (44) focused on a significant damage in the microvasculature within the kidney scaffold, which resulted in the cessation of blood flow. This thorough investigation was necessary to accurately evaluate the vascular integrity of the kidney scaffold.

*(vii) liver fibrosis:* The authors of reference (45) postulate that α-mangostin (AM), the major constituent of the xanthone fraction in extracts of *Garcinia mangostana* L., may protect the hepatic microvascular bed from thioacetamide (TAA)-induced fibrosis.

From this short summary of ongoing research topics in biomedicine, where the vascular corrosion casting technique was applied, it can be concluded that this method is still of great importance and leads to highly qualitative results where other imaging methods of the blood vascular system, such as angiography or even μ-CT, reach their limits. Even though, many of these studies would further benefit from additional quantitative analyses of vascular casts (3D morphometry) (10).

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