Three-dimensional computer reconstruction of the airway- and the vascular systems of the lung of the domestic fowl, *Gallus gallus* variant *domesticus*

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Abstract

Invariably, any type of microscopic imaging modality can be used to reconstruct morphological data in three-dimensions (3-D). These techniques allow us to study the structural characteristics of cells, tissues and organs and gaining meaningful insights into their form and function. One of the oldest methods for studying biological entities in 3-D is serial histological-section reconstruction (SHSR) which dates back to the late 19th century. Despite the advent of competing modern techniques that are faster, cheaper and easier to apply and that work in an automated fashion, this method (SHSR) remains indispensable. It is because reconstructions resulting from sections generally display better contrast and signal-to-noise ratio. Additionally, dyes and labels can be used more effectively when applied to the surfaces of sections than to, e.g., resin sample blocks: functional units can therefore be identified by coloured- or fluorescent signals. SHSR is useful from ~0.2 µm resolution and can easily sample large volumes in the 1000^3 voxel range. These characteristics make it particularly effective for preparing accurate, high-resolution 3-D representations of micro-anatomical data. Here, we report on computational use of SHSR in investigation of the structure of the airway- and vascular systems of the mature lung of the domestic fowl, *Gallus gallus* variant *domesticus*. The topographical relationships between the structures were thoroughly examined after preparation of movies that allowed us to rotate the reconstruction around different axes and extract and re-insert different parts back into the visualization. In the extant air-breathing vertebrates, the avian respiratory system (the lung-air sac system) is structurally the most complex and functionally the most efficient. Its unique design largely explains how, among only a few other extant animal taxa, birds attained volancy. Powered (active) flight is an exceptionally energetically costly mode of locomotion which requires large amounts of oxygen to sustain. While it has been continuously studied for over the last four centuries, some aspects of the structure and function of the avian lung remain uncertain and contentious. They include the airflow dynamics across the lung, the arrangement of the airway- and vascular systems and the shapes, sizes and spatial organization of the terminal respiratory units. We noted that in contrast to the mammalian lung, where arterial- and airway systems track each other and the venous system runs segmentally, i.e., between the other two systems, in the avian lung, the corresponding structures do not display similar close following. This may be explained by the unique development and evolution of the avian lung: two morphologically- and functionally distinct parts, namely the paleopulmo and the neopulmo, exist. The parts develop at different times which later combine into one organ. It explains how and why the airways- and the blood vessels do not track and even pattern each fundamentally because the conduits are dedicated to the distinctive parts of the lung with which they were associated.

Introduction

A number of techniques are available to generate volumetric ultrastructural models, and combination of a variety of strategies is now possible for tailoring to specific biological questions and applications. [1]

Through the process of evolution by natural selection [2], living things develop in specific ways to enable them to best execute functions. Understanding cellular dynamics and processes is fundamental to characterizing the underpinnings of life. Most diseases and pathologies arise from cellular abnormalities which trigger intracellular biochemical changes. After the invention of the compound light microscope, some four-and-half centuries ago by Zacharias Jansen (1580-1638) in 1595 [3] [4] and for nearly three hundred years thereafter, microscopic study of biological structures was limited to two-dimensional (2-D) delineation, depiction and description. In the late 19th century, researchers desiring to understand the three-dimensional (3-D) attributes of biological entities employed different ingenious visualization aids to examine serial sections, such as projecting lines from the outlines of structures to produce an image of the object perpendicular to the direction of sectioning [5] or by tracing outlines of the object of interest onto stacks of glass sheets [6] [7]. Modifications on these types of techniques were still being used [8] when Sydney Brenner first applied computers to the problem of visualizing serial section reconstructions in the early 1970's [9]. More recently however, advances in computing power; software; imaging technology as well as visualization tools have spurred the development of a large number of 3-D reconstruction approaches. These techniques are revolutionizing the fields of molecular-, cellular- and tissue biology [1] [10]-[21]: even four-dimensional (4-D) preparations or visualizations are now possible [21]-[23]. The distinctive functions that are displayed by biological structures stem from a multitude of minute physical and biochemical events than occur between and among topologically connected structural components at the different levels of the organization of highly complex assemblages [24]. The spatial conformation (shape) of the structural components that comprise living matter is ubiquitously 3-D [25]-[28]. Recently, 3-D cell- and organoid culture have become particularly popular in studies of cell development, morphology, differentiation, host-pathogen interaction and effects of drug treatments compared to the traditional 2-D ones [23] [29]-[41]. It has been recognized that cells which are cultured in 3-D cell culture assays behave utterly differently compared to those 'grown' in 2-D ones [31] [32]. Comparative investigations have indicated that 2-D cell cultures lose tissue distinctive assemblage, generating changes in mechanical and biochemical signals and disrupting cell-cell or cell matrix connections [31]. In drug delivery studies, while many preparations may show success in 2-D cell cultures, this is not the case in *in vivo* studies because the 3-D environment of the large number of cells in the body may render it problematical for the drugs to equally affect all cells [30]. Furthermore, although in 2-D culture cells acquire a spheroid morphology, the clusters are formed mainly by aggregation of cell masses instead of dividing from a single cell and the groupings of cells are significantly larger than those obtained by 3-D culture [36]. 3-D cell culture optimally supports the 3-D shape of cells, thereby providing a physiologically appropriate environment similar to the one which exists in the living tissue [34]-[36]. It is by coiling and folding into proper 3-D shape that proteins can perform their complex biochemical function(s). Here, two examples are given to underscore the importance of 3-D configuration in proper function of biological components.

In microscopic biology, 3-D reconstruction is a highly instructive investigative technique [18] [42]-[45]. It involves constructing a spatial model of a biological entity from a sufficient

number of 2-D images [46]-[48]. Unfortunately, because of the high cost of time and materials as well as the special skills required to perform it, 3-D reconstruction has not been employed in morphological studies as much as it should have been. The 2-D images needed to prepare the 3-D shape can be acquired by manual preparation of serial sections or by tomographic methods using any measureable signal that is able to penetrate the specimen such as: light, ultrasound, gamma rays, X-rays, neutrons or electrons [18] [49]-[52]. Serial section 3-D reconstruction is the oldest method of obtaining 3-D spatial micro-anatomical data and dates back to the late 19th century [53]-[55]. Recent advances in optical microscopical sectioning techniques as well as automatic block-face image capturing methods like episcopic fluorescence image capturing (EFIC) or surface imaging microscopy (SIM) [56] [57] have considerably increased the efficiency and the accuracy of serial sectioning. Regarding possible sub-micron resolution, compared to the modern 3-D reconstruction techniques, conventional light microscopical (histological) sections have certain advantages of which the main ones are contrast and large sample size which is investigated [11] [18] [58] [59]. Only a few state-of-the-art 3-D reconstruction techniques, e.g., synchrotron-based microcomputer tomography (µCT) or focused ion beam scanning electron microscopy (FIB/SEM) tomography compare in resolution with imaging sections [60]. As more robust ways of utilizing 3-D reconstruction technology continue to be developed, 3-D imaging and animation will constitute an indispensable investigative arsenal in the morphologist's toolbox. Old questions will be revisited and investigated from different perspectives and new questions will be formulated and researched. 3-D reconstruction has lately found application in new research fields such as tissue engineering and regenerative medicine [60]-[63].

In this investigation, we have performed 3-D computer reconstruction on serial histological sections to study the spatial arrangement of the airway- and the vascular systems of a bird's (avian) lung. Among the extant air-breathing vertebrates, the avian respiratory system (the lung-air sac system) is structurally the most complex and functionally the most efficient [64]-[72] compared with the mammalian one. Although it has been continuously investigated for well over four centuries (i.e., since [73]), still, some important aspects of its biology remain unclear and/or controversial [74]. Like the invaginated gas exchangers of other vertebrates, the lungs of birds are ventilated tidally, i.e., in-and-out, and in addition the avian lung (specifically the paleopumonic part of it) is ventilated unidirectionally and continuously in a caudocranial direction, i.e., back-to-front. This is achieved by synchronized bellows-like actions of the air sacs. The path followed by the inspired air across the avian lung is controlled by aerodynamic valving [75] and not by anatomical valves or sphincters, as it was once believed. Morphometrically, the avian lung has thin blood-gas barrier, large respiratory surface area and large blood capillary volume, structural parameters which confer an exceptionally high pulmonary diffusing capacity for oxygen [71] [76]-[78]. Such specializations explain why except for bats, birds are the only other vertebrate animals that have attained powered (active) flight which is an energetically highly costly form of locomotion which requires particular specializations [79]. Some birds can fly nonstop over long distances and others fly under the extreme hypoxic conditions of the high altitude [78]-[82]. Here, we present a 3-D reconstruction method for multi-view image acquisition of microscopic samples combined with pre- and post-processing steps including correlation-based image registration, filtering and a combination of manual and automated segmentation.

Materials and methods

Fixation and processing of the lung

The Animal Ethics Committee of the University of Johannesburg approved all experimental procedures (Clearance Number: 2017-06-29/Maina). A mature domestic fowl (chicken), *Gallus gallus* variant *domesticus*, was killed by intravenous injection with pentobarbitone sodium (Euthanase®) into the brachial vein at a dosage of 2mg/kg. Thereafter, the lungs were fixed by intratracheal instillation of phosphate buffered 2.5% glutaraldehyde (350 mOsm L^{-1}, pH 7.4) at a pressure head of 3 kPa. The trachea was ligated and the fixative left *in situ* for six hours. Afterwards, the lungs were carefully dissected out from their costal attachments. Whole lungs were processed and embedded in paraffin wax according to routine histological procedures.

Serial sectioning and imaging

Two-thousand six hundred and eighty-nine (2689) transverse serial sections were cut at 8 µm thickness, stained with haematoxylin and eosin and mounted onto glass slides. The whole series of sections constituted the entire lung volume. A total of thirty seven (37) sections were lost or damaged during sectioning. The rest were mounted onto glass slides. In most cases, the lost sections were non-consecutive, but in one case nine sections (72 µm) were lost in a row. An area measuring 12.88 × 9.655 mm, which included the entire transverse section through the lung was photographed using an Axioskop image analyser (Zeiss Instruments) at a magnification of x10 in uncompressed Tiff image format at a resolution of 2576 × 1931 pixels with a calibrated sampling of 5 µm/pixel.
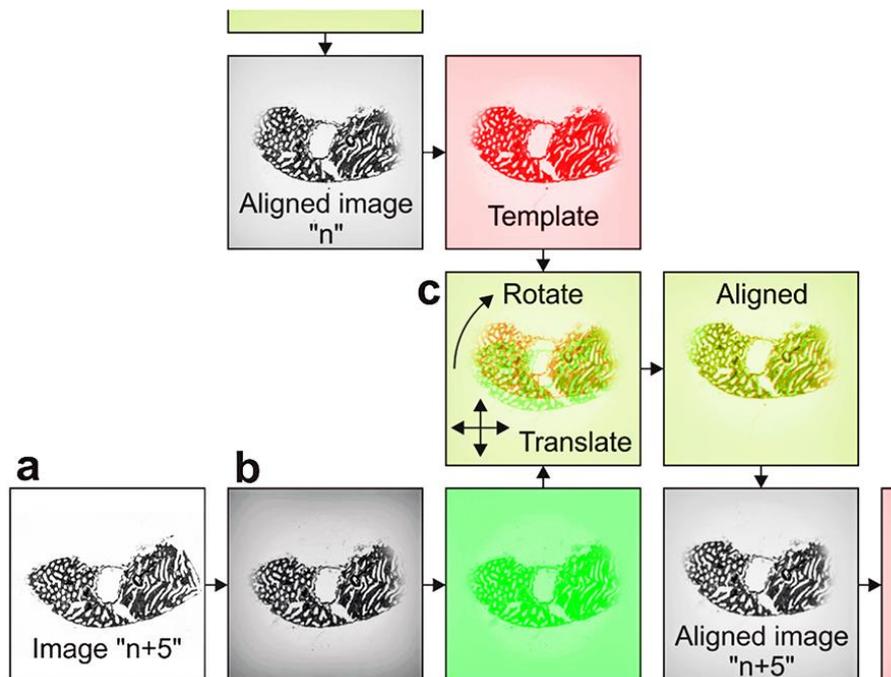
Figure 1: Image processing and alignment. (a). Every 5th image was selected, normalised and down-sampled. (b). The images were multiplied by a mask function to prevent the image border and particles of dust from contributing to the alignment score. (c). The previously aligned image ("n") is used as a template and the images are correlated with one another: this is illustrated using red/green. The image to be aligned (n+5) is rotated and translated relative to the template (n) until a maximum correlation score is obtained (aligned). This newly aligned image is then used as a template to align the next image (n+10) and the process is repeated.

Image alignment

Every fifth image was selected. In cases where this section was missing or showed obvious defects such as folds, tears, compression or inadequate staining, the previous- or subsequent section was chosen. This produced five hundred and thirty five (535) images (representing 40 µm in the Z-direction), which were manually corrected in brightness and contrast using ImageJ Version 1.4.0. [83] and imported into Spider V.15 [84]. Images were normalised to a mean of 0 and standard deviation of 1 and down-sampled by a factor of 8 using bicubic interpolation to yield a sampling of 40 µm in X and Y (Fig. 1a). The images were then multiplied by a mask function representing a Gaussian falloff (Fig. 1b) and aligned to one another by maximising the cross-correlation function in X, Y and in-plane rotation (Fig. 1c) [12]. The resulting alignment was sufficient for resolving the parabronchi and larger blood vessels (Fig. 2) and permitted satisfactory reconstruction.
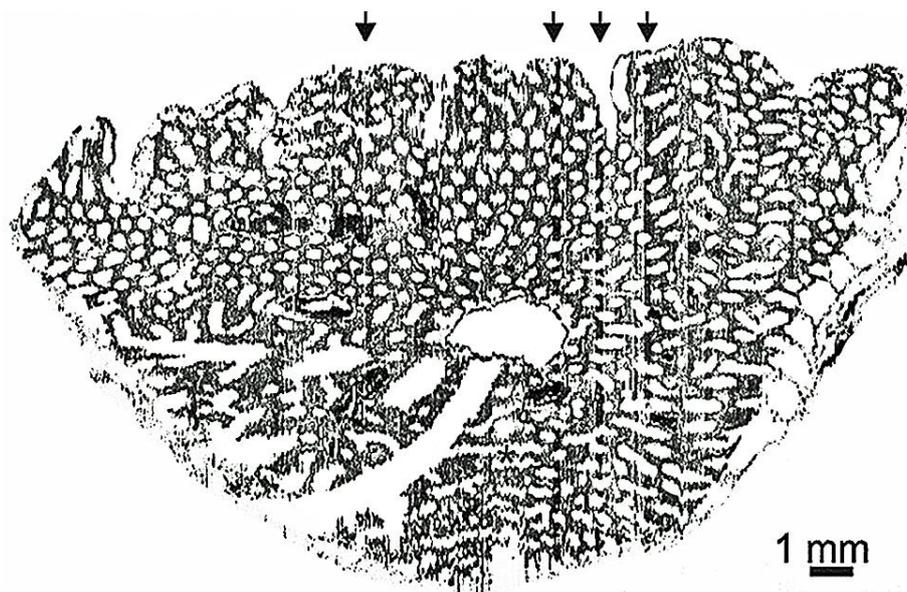
Figure 2: A cross-section (image 127) of the lung through the aligned image stack. All 535 individual sections can be seen as vertical lines. Errors in vertical alignment can be seen as "jitters" in individual structures. Areas where this is especially apparent are marked (*). Large differences in intensity between adjacent sections can be seen as vertical bands (arrows).

Segmentation of the reconstruction

To identify and segment the air-conducting elements of the reconstruction, an automated procedure was used. It involved applying a Gaussian filter and a threshold [12] [14]. In some cases, where the parabronchi were separated from adjacent ones by interparabronchial septa, a border was manually drawn around the periphery of the lung. Also, where two air-conducting elements lay adjacent to one another, to ascertain that they were resolved, lines were manually drawn between them. This process was iterated until a reasonable match between the unprocessed images and segmentation [85] was achieved (Fig. 3).

Blood vessel segmentation was achieved by manually defining the border of each blood vessel in the original (unprocessed) colour images (Fig. 3). These images were then down-sampled and aligned to one another by applying the alignment parameters obtained previously. To ensure that no blood vessels were mistaken for air-conducting elements

(especially those lacking erythrocytes in their lumen), the segmented blood vessels were subtracted from the air-conducting elements.

Reconstruction processing and display

The above procedure produced three image stacks: "air", "blood" and "original images". These were converted into volumes using Spider V.15 and low-pass Fourier filtered to a resolution of 160 µm with a Gaussian falloff [84] [85]. All three reconstructions were simultaneously displayed in UCSF Chimera 1.12 [86], the appropriate surface threshold value being determined by comparison with the "original images" volume. Larger blood vessels and airways were visualised by applying an additional low-pass Fourier filter to a resolution of 0.8 mm and adjusting the threshold value. The segmentation function in Chimera was used to further segment the blood vessels into arteries and veins.
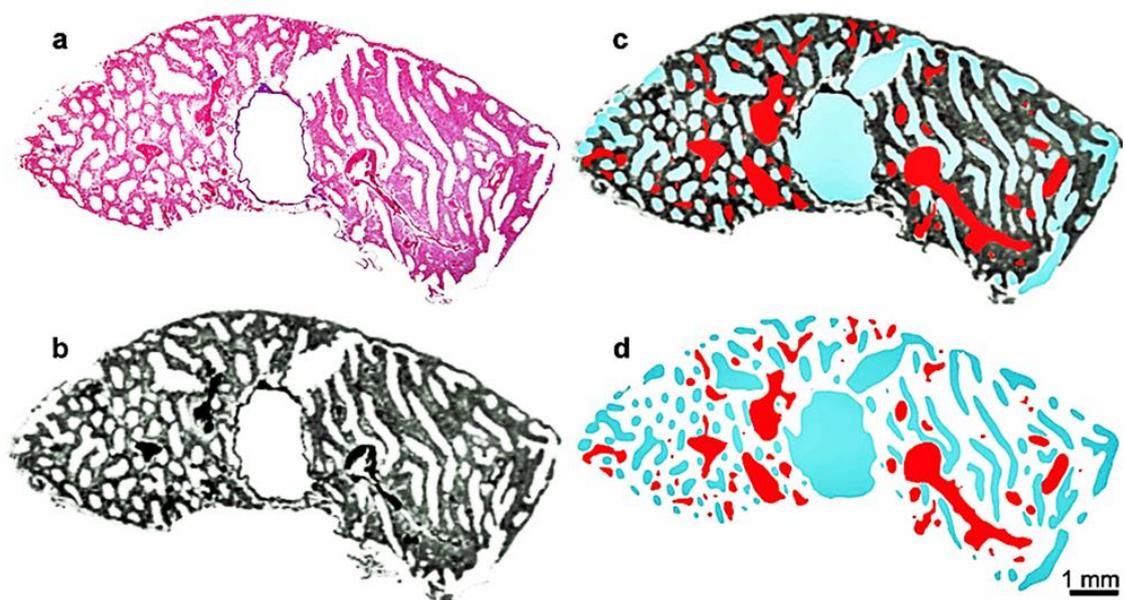
Figure 3: Segmentation of the reconstruction. (a). An unprocessed hematoxylin and eosin stained image (section 233) showing various air-conducting elements and blood vessels. (b). The same section shown in (a), but resampled, normalised and aligned. The larger vessels and lumina of the parabronchi are sufficiently well-resolved. (c). The manual blood vessel segmentation (red) and automated airways segmentation (cyan) superimposed on (b). An excellent match between the structures identifiable in the section was obtained. (d). The final image showing the airways (cyan) and blood vessels (red).

Results

Airways: bronchial system

The trachea divided into right- and left extrapulmonary primary bronchi (EPPB) at the syrinx. The EPPB penetrated the lung at the hilus where they lie craniolateral to the pulmonary artery (PA) and caudomedial to the pulmonary vein (PV). On entering the lung, the EPPB becomes the intrapulmonary primary bronchus (IPPB) or the mesobronchus which changes in diameter and course as it passes through the lung to exit the lung at the abdominal air sac. On the various aspects of its lumen, as it passes through the lung, the IPPB gives off four sets of secondary bronchi. These are: the medioventral secondary bronchi (MVSB) that originate from the dorsomedial aspect of the lumen; the mediodorsal secondary bronchi (MDSB) that arise from the dorsal wall; the lateroventral secondary bronchi (LDSB) that arise from the caudoventral part and; the laterodorsal secondary bronchi (LDSB) that emanate from the

lateral aspect of the distal part of the IPPB. The parabronchi or the tertiary bronchi interconnect the secondary bronchi. The parabronchial system, which connects the MVSB to the MDSB, forms the paleopulmo or the 'old lung' while those which connect the MDSB to the LVSB and the LDSB form the neopulmo or the 'new lung'. The paleopulmonic parabronchi form a stack or pile of air conduits which largely occupy the dorsocranial part of the lung while the neopulmonic ones are mostly located on the caudoventral part of the lung. As they (paleopulmonic parabronchi) join the MVSB to the MDSB on the dorsal aspect of the lung, they form hoop-like shapes: the paleopulmonic parabronchi lie parallel to each other and sporadically anastomose with each other while the neopulmonic parabronchi anastomose profusely, forming a dense network. Generally, the paleopulmonic parabronchi are larger in size compared to the neopulmonic ones.

Vascular systems: pulmonary artery (PA)
The PA enters the hilus ventral to the root of the first MVSB. On penetrating the lung, it divides into four main branches (= rami), namely the accessory-, the cranial-, the caudomedial- and the caudolateral branches. The branches supply blood to different parts of the lung: the accessory branch, which is the first blood vessel to arise from the PA, supplies blood to a small part of the lung ventral to hilus; the cranial branch supplies blood to the craniodorsal region of the lung cranial to the second costal sulcus; the caudolateral branch supplies blood to the ventrolateral part of the lung and; the caudomedial branch, which is the most direct extension of the PA, supplies blood to most of the lung caudal to the second costal sulcus. The four branches of the PA divide the lung roughly into a cranial- and a caudal arterial vascular region, with a vertical transverse line passing through the second costal sulcus forming the anatomical landmark or the dividing boundary: the cranial part of the lung is supplied with blood by the accessory- and the cranial branches while the caudal one is supplied by the caudomedial- and the caudolateral branches. Along the median longitudinal plane which divides the lung into a lateral and a medial half along the so-called *linea anastomotica*, i.e., the area marking connection between the parabronchi from the MVSB and those from the MDSB (the paleopulmonic parabronchi) meet, the former part of the lung is supplied by the caudolateral branch of the PA while the later one is supplied by the caudomedial branch. In this study, up to about the level of the interparabronchial arteries, no anastomoses were observed between the four branches of the PA.

Vascular system: pulmonary vein (PV)
At the hilus, the PV is separated from the PA by the intrapulmonary primary bronchus. The PV is formed by connection of three converging blood vessels (radices), namely the cranial-, the caudal- and the ventral radices. The radices join outside the lung to form the PV. In our study, the connection of the radices was not included in the 3-D reconstruction because that part of the lung was inadvertently cut off during trimming off of adhering connective tissue. Most of the craniodorsal part of the lung is drained by the cranial radix which is formed by confluence of three large veins; the caudal radix drains the part of the lung caudal to the third costal sulcus and is formed by as many as four radices which extend dorsally and ventrally and; the ventral radix, which drains the cranioventral part of the lung comprises two main branches which drain the part of the lung located between the second and the third costal sulci. Up to the level of the interparabronchial veins, no anastomoses were observed between the radices.

Discussion

Employing different methods, the morphologies of the airways of the avian lung have been investigated by various investigators [64]-[66] [71] [87]-[90]. Consensus on the numbers of

airways, their shapes, connections, topographic locations and nomenclature has, however, not yet been reached [74. While interspecific differences have been reported [64]-[66] [68] [71] [91] [92], it cannot be completely ruled out that interspecimen differences occur largely from developmental abnormalities and irregularities and the environmental conditions under which the avian eggs are incubated. In this study, in addition to 3-D reconstruction of the airways- and the blood vessels, for more insightful visualization of the morphologies and the spatial associations between the different parts, movies which allowed the rotation of the constructions across different planes and extraction (removal) and re-introduction of the different parts to the constructions were prepared. Although certain weaknesses exist in all the techniques which have been used to study the morphology of the avian lung, this study corroborates most of the previous accounts. Indisputably, 3-D reconstruction is a very powerful means of studying the morphologies of biological structures, including those of structurally complex avian respiratory system.

The pulmonary vasculature of birds has been studied by [93]-[98]. Various techniques which included injection with markers and following the paths they follow, e.g., microspheres, in the blood vessels by light microscopic examination and microfilm, silicone, mercox or latex rubber injection followed by maceration and preparation of casts or replicas have been used. While our observations generally agree with those previously made by other investigators, certain differences exist. Unlike in the mammalian lung where arteries closely follow airways while veins run intersegmentally [99] [100], i.e., they are located between the airways and the arteries, in the avian lung, the airway- and the vascular systems do not display such arrangement. This may be explained by the complex development of the avian lung [101]-[103], where, in lungs of phylogenetically derived species (evolutionarily advanced birds), the paleopulmonic part develops first to be followed by the neopulmonic one [102] [103]: the adult lungs of an evolutionarily developed bird comprises two distinctive parts, namely the 'paleopulmo' and the 'neopulmo' which display structural and functional differences: the two regions are typically located in different regions of the lung [64] [68] [92] and the two parts are ventilated differently. The paleopulmo is continuously and unidirectionally ventilated in a caudocranial direction by concerted actions of the air sacs [67] [104] while the neopulmo is ventilated tidally, i.e., back-and-forward. Taking these properties into consideration, it is axiomatic that the airways and the blood vessels of the avian lung cannot follow each other in the same way as in the mammalian lung, where mesenchymal cells which contribute to the development of the airways and the blood vessels start at the same point (essentially the lung bud) and in close proximity grow outwards as the lungs develop [105]-[107], forming the various functional systems. Regarding the observations of [93] [94], in comparison with the observations noted in this study, certain structural discrepancies exist. The main ones are: a) while two main radices were reported to converge and form the PV [93] [94], here, three main blood vessels drained the lung and joined to form the PV and; b) the second costal sulcus and not the third one formed the boundary between the cranial- and the caudal blood supply- and the respective drainage regions of the lung by the PA and PV, respectively.

In conclusion, compared to the other techniques which have hitherto been employed to study the morphology of the airway- and the vascular systems of the avian lung, incontrovertibly, 3-D reconstruction is the more robust technique. When it is combined with preparation of movies which can be operated and closely viewed from different angles, the geometries of the structures can be thoroughly scrutinized and understood. It is important to underscore that while a powerful technique in its own right, scanning electron microscopy (SEM), which is conventionally applied for imaging biological structures, does not strictly generate 3-D images as generally wrongly interpreted: although they may appear so, the resulting images

do not have an aspect of depth. Presently, there are several SEM techniques that can be used to obtain 3-D information on a biological sample [108]-[110]. Some of them can be done on any microscope and some require specialist instrumentation, software, or microscopes.

Acknowledgements

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