Angiofil: a novel radio-contrast agent for post-mortem micro-angiography

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ABSTRACT

The radio-contrast agent Angiofil has recently been developed to be predominantely applied in forensic medicine. Angiofil is a liquid radio-contrast agent based on iodine. Its viscosity is easy to adjust by the choice and the concentration of the solvent. Therefore, it is well suited for penetrating vessels of different diameters. The liquid Angiofil avoids the sedimentation of suspensions containing radio-opaque materials such as barium sulfate. The injection of Angiofil into the vascular system of mice post-mortem results in remarkable data showing the vascular trees of tissues and entire organs. Penetration into the surrounding tissue was not observed. Consequently, Angiofil has the potential to reach the performance of the established casting agent Microfil.

Keywords: Angiofil, radio-contrast agent, vascular tree, vascular phenotyping, micro computed tomography, micro-angiography

1. INTRODUCTION

The biomedical imaging market is currently seeking a steady growth. One large field comprises the development and marketing of novel radio-contrast agents in order to enhance the imaging capabilities. The visualization of the vascular systems of tissues, organs or entire small animals becomes more and more important. In particular, the detailed morphological analysis of the vascular tree allows assessing the basic physiological conditions and metabolic functions. Filling vessels with radio-opaque materials has been established. The application of several compounds have been reported.[1] Apart from the lyophilic salts, such as barium sulfate, iodine compounds were used because of its high x-ray absorption. A powerful but time-consuming alternative is the filling of blood vessels with resins, often methyl methacrylate. After polymerization the tissue can be dissolved and a fragile construct forms that can be easily imaged.

The present paper describes the performance of the lipophilic radio-contrast agent Angiofil in micro-angiography and micro computed tomography. Based on applications in forensic medicine, the contrast agent has been patented.[2] Experts in bio-imaging have evaluated Angiofil within an international framework. The initial study[3] included the injection into ten mice for micro-angiography post-mortem. Whole body scans at isotropic 40 μm pixel size should permit to make visible the vascular systems. It has to be uncovered if the dissection of individual organs really facilitates the segmentation of the micro-vessels. Parameters such as vessel length and caliber should be quantified. Color-coding should help to obtain detailed 3D representations of morphology of the vascular system.

Furthermore, Angiofil has to be comparatively evaluated with respect to established staining protocols. Such a comparison should include barium sulfate that is known to be effective for lung vessel imaging in rodents.[4] In addition, the performance of Angiofil should be compared to casting procedures based on Microfil.

Another important issue to introduce Angiofil into the market is the handling. The procedure has to be relatively easy and fast. The data quality has to be good enough that data processing such as intensity-based segmentation yields three-dimensional images comparable to the casting. Finally, the waste management has to be considered.

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Developments in X-Ray Tomography VI, edited by Stuart R. Stock,
Proc. of SPIE Vol. 7078, 70781O, (2008) · 0277-786X/08/$18 · doi: 10.1117/12.792077

2. MATERIALS & METHODS

Angiofil consists of two liquid components, namely the iodized oil (alkylesters of fatty acids) and the dedicated diluent. The oil gives rise to high contrast of more than 2000 HU. Adding the solvent, one can adjust the viscosity of Angiofil. In the present experiments, the iodized oil and the diluent were mixed with a ratio of 1:5. The related viscosity corresponds to 3.4 mPas at the temperature of 20 °C.

For the initial study, ten wild-type mice were anesthetized applying the intra-peritoneal injection of ketamine and xylazine. Angiofil was manually injected at room temperature following two protocols.[3] Upon fixing and complete perfusion, the main afferent and efferent vessels of the organs were ligated, before the organs were removed and placed in a Petri dish to be immersed in paraformaldehyde. The specimens in the dishes were scanned by the micro CT system (ForBild scanner, Institute of Medical Physics, Erlangen, Germany) using an accelerating voltage of 40 kV and a voxel size of 15 µm. The data were processed on a Leonardo workstation (Siemens). The merging of partial body scans and the building of the 3D models were done by means of the Amira software package (Mercury Computer Systems). Color-coding of vessel caliber and the subsequent remapping onto the surface of the 3D model were performed as previously described.[5]

For the barium sulfate staining, two female nude mice (Balb/c, weight about 25 g) were anesthetized using isofluorane. The contrast agent was injected into the tail vein of the mouse. Here, the suspension of 15 g barium sulfate in 200 ml physiological sodium chloride solution was used. This suspension was filtered (pore size 40 µm, BD Falcon, USA) in order to inject 500 µl that only contained particles with diameters of the micro-vessels. Just before injection, the suspension was mixed for a period of 10 min in an ultrasound bath (Sonorex Digital 10P, Bandelin) at the temperature of 37 °C. Furthermore, the liquid mixture of 20 µl Angiofil and 60 µl solvent was applied in the same manner.

In the case of the barium sulfate suspension, the mouse survived for 5 min until it was sacrificed. In the case of Angiofil staining, the mouse died within 2 min upon injection because of the non-physiological solvent used. Several organs, namely lung, brain, liver, kidneys and spleen were isolated and fixed in paraformaldehyde for experiments at the synchrotron radiation sources at HASYLAB/DESY, Hamburg, Germany, which have been operated by the GKSS Research Center.

The barium sulfate stained lung, held in an Eppendorf container in liquid, was scanned at the beamline W 2 using the photon energy of 38 keV, a value just above the K absorption edge of barium (37.44 keV). The pixel size corresponded to 6.8 µm. The spatial resolution of 13.7 µm was determined by means of the modulated transfer function of a highly x-ray absorbing metal plate at the sample position. The tomograms were obtained by means of the standard filtered back-projection reconstruction algorithm out of 720 projections. The 3D dataset of the lung was generated combining five tomograms at different height levels each with voxel precision.

The Angiofil stained lung was scanned in similar fashion at the beamline BW 2 using the photon energy of 17 keV and the pixel size of 7.0 µm.

For Microfil experiments, female C57/BL6 mice were anesthetized with an intra-peritoneal injection mixture of ketamine and xylazine, and a subcutaneous injection of 500 units of heparin was given. A midline incision was made and the heart exposed. A catheter was inserted into the left ventricle and 1X PBS was allowed to flow in. The right atrium was then cut for drainage and the flow of PBS continued at a constant pressure of 100 mmHg until the fluid exiting the atrium was clear and the organs were uniformly blanched. Then Angiofil (5 ml, 1:5 mixture contrast agent : solvent, v/v) was infused at 150 mmHg constant pressure. Upon clamping the tubing line, the vessels were carefully ligated and the organs were removed. For perfusion with Microfil silicone rubber injection compounds (MV-122, Flow-Tech Inc.), a mixture of compound, diluent, and catalyst was infused at 160 mmHg pressure. Upon clamping the tubing line, Microfil was allowed to polymerize during more than 90 min. Organs of interest were carefully removed. Upon dissection, specimens were stored in neutral buffered formalin, and subsequently mounted in 1% agar for micro CT imaging on a GE eXplore Locus SP specimen scanner. Kidneys were scanned at the accelerating voltage of 80 kV and a beam current of 80 µA using the isotropic 20 µm pixel size.

3. RESULTS & DISCUSSION

3.1 Imaging vasculature with the contrast agent Angiofil

The benefits, gained from the analysis of vascular systems by micro CT, have been demonstrated. This included the putative advantages and disadvantages of the contrast agents.[6] Angiofil should fit well into the list of candidates for blood vessel imaging. Thus, it has the potential to become a valuable additive for vascular phenotyping. Imaging the vascular systems in whole body mice as well as in individually dissected organs by means of Angiofil gave rise to remarkably detailed overviews of the main vessel trees, as recently published.[3] Within this study, the tomograms of the partial body scans were merged to represent the entire vascular system in the 3D manner. By adjusting the region of interest, the main vessels of the vascular systems of different organs became visible and were color-coded according to their caliber size. Vascular branches of the liver, vessels of spleen and kidneys were clearly detectable. The heart was completely filled with the contrast agent. So, cardiac and pulmonary vessels became partially visible. High-resolution scans of individual organs revealed the micro-vessel morphology in detail. Within this study, the small arteries and veins of the liver and the kidneys and even the capillaries of the lung were visualized down to the pixel size of 15 µm. Since selected cross-sections were difficult to be evaluated, maximum intensity projections were generated for further analysis. They gave a comprehensive overview of the vascular structure as demonstrated for the kidney in Fig. 1.

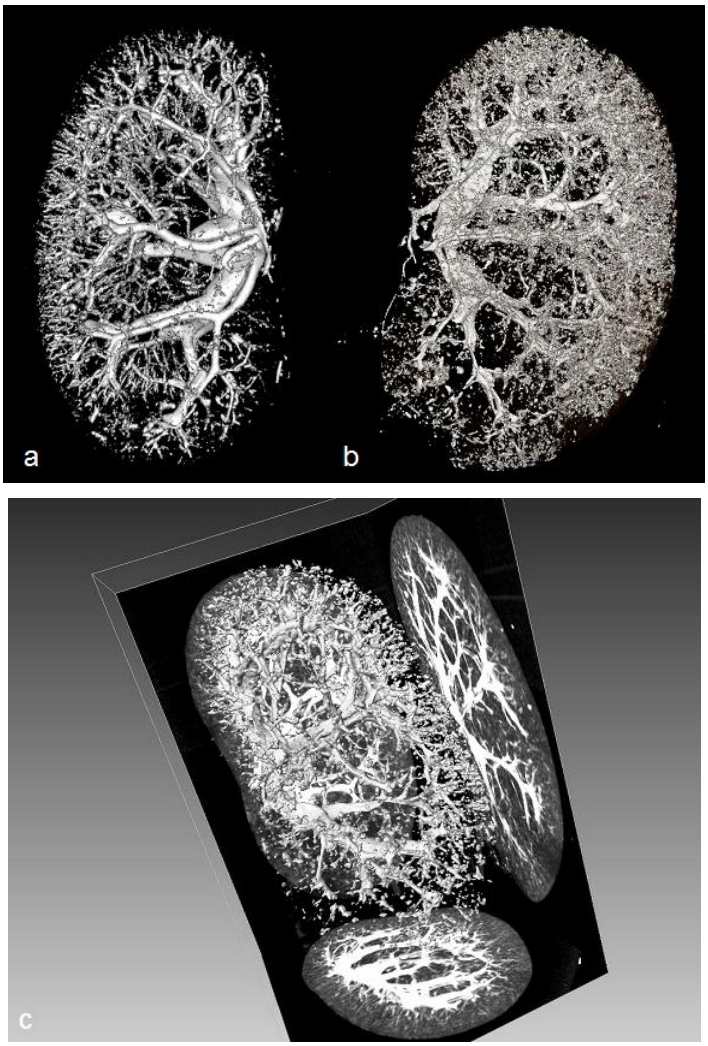

Fig. 1. (a) Representations of a high-resolution scan of a murine kidney visualizing the interlobular vessels in detail. (b) After adjusting the region of interest, the smaller vessels of the cortex and of the glomeruli become included. (c) Using the software Amira, the maximum intensity projections on the cube side create the iso-surface rendering of the vascular surface.[3]

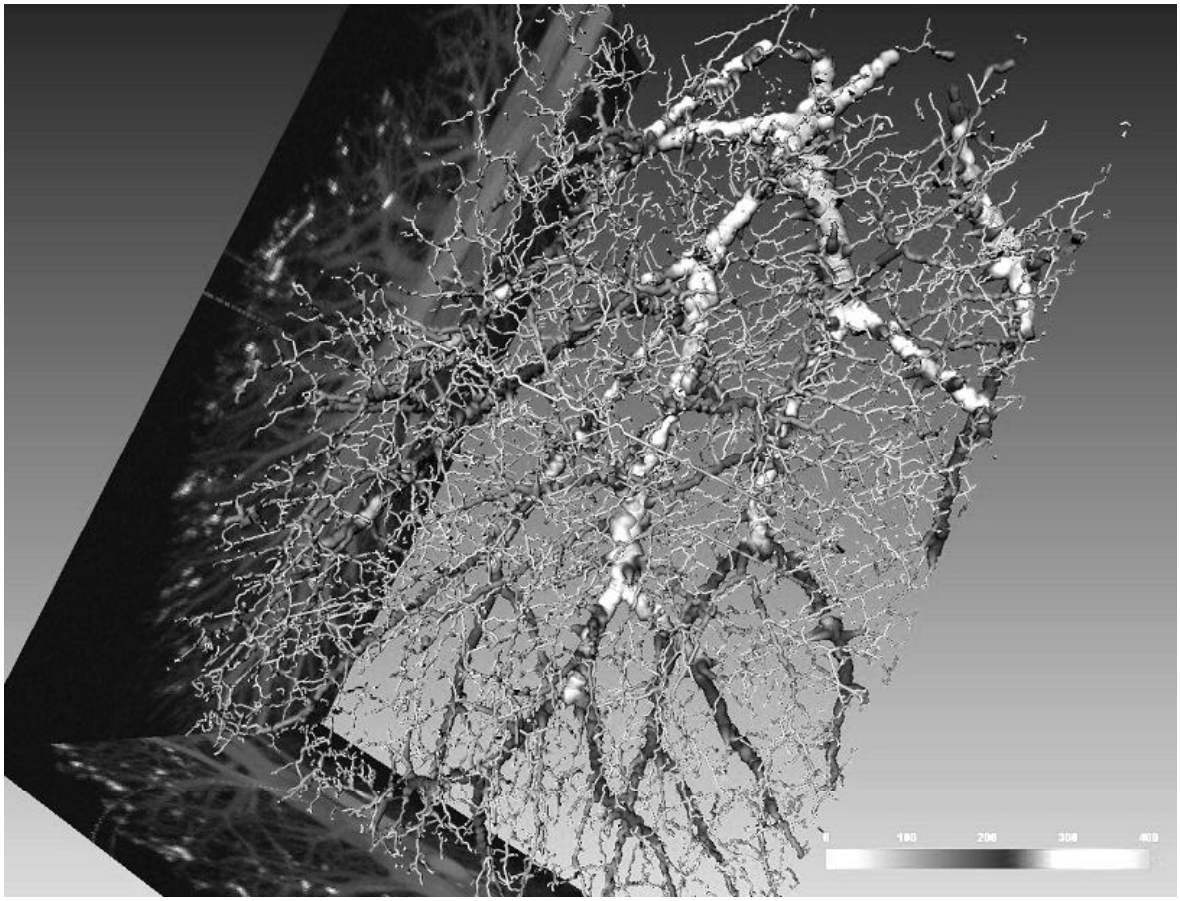

Fig. 2. The high-resolution data of a part of the murine liver show the vascular tree with gray-value coded vessel calibers.[3]

Fig. 2 shows the high-resolution liver data. The vessels were quantified and remapped onto the surface of the vascular system according to their caliber, i.e. from light gray via dark gray to white. Light gray is used for thin vessels with mean diameter between 50 and 100 µm, The dark gray corresponds to vessels with a mean diameter of 300 µm, whereas the thickest vessels 400 µm wide in average are represented in white.

Angiofil stayed within the vessels for a long time. Thus, it is well suited for angiography. It does not penetrate through the vessel walls. Monitoring Angiofil in the chemically fixed tissue, it remained stable for days without detectable leakage.[7] In the present study, we investigated organs more than one week after their perfusion with Angiofil, and did not find any deterioration in image quality.

The vessels filled with Angiofil exhibit much higher x-ray absorption than the surrounding tissues. This high difference in absorption facilitates the automated segmentation of the vessel tree that is necessary to extract quantities such as vessel length, caliber and bifurcation density. The intensity-based segmentation is also the first step to apply more sophisticated tools for erosion/dilatation, distance mapping, etc. In order to identify the morphological features the chemically fixed tissues is amenable to histological analysis.

3.2 Comparison of Angiofil and barium sulfate staining

The main difference between Angiofil and barium sulfate is the state of aggregation at the application temperatures. The relatively heavy, insoluble barium salt is solid and gives rise to sedimentation phenomena, which finally lead to the inhomogeneous distribution within the vessels. Therefore, the vessels often appear disconnected.[8] Angiofil is purely liquid. Hence these sedimentation problems are avoided. The lung, however, can ‘filter’ the micrometer-sized barium sulfate particles. This filtering effect results in high barium concentrations in the lung, which yields high-contrast images as shown in Fig. 3. Angiofil staining of the lung leads to comparable images (cp. Fig. 3).

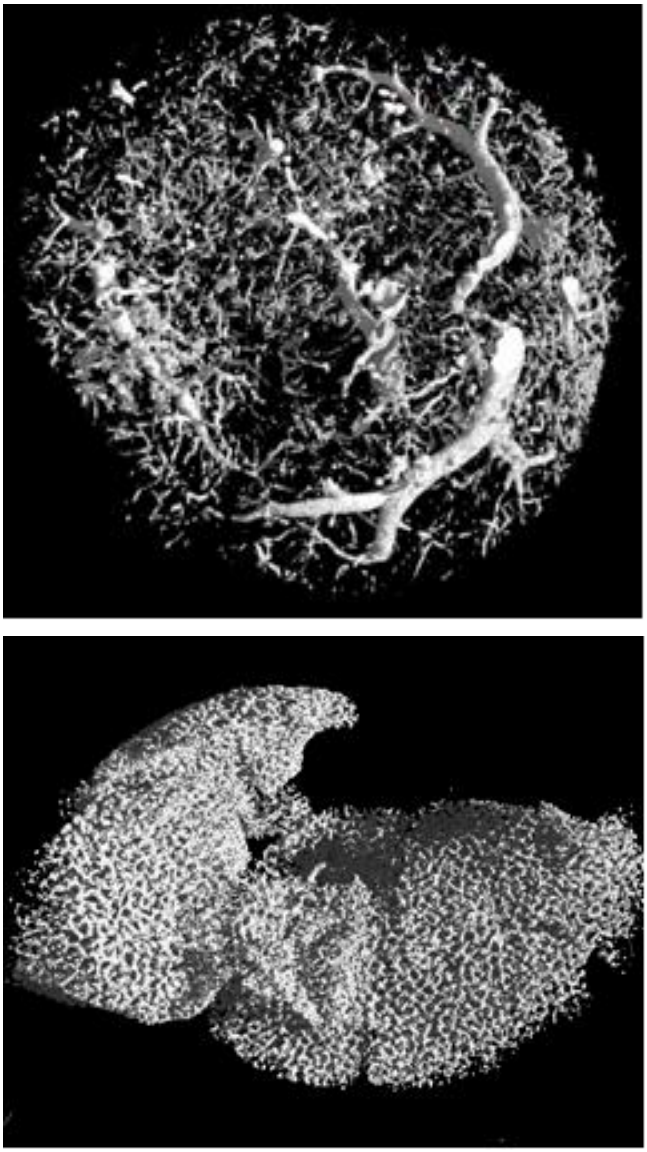

Fig. 3. The 3D visualizations of mouse lungs perfused with Angiofil (top) and barium sulfate micro-particles (bottom) are based on synchrotron-radiation based micro CT and generated by the software VGStudio Max 1.2 (Volume Graphics, Heidelberg, Germany).[4]

The mouse lungs are well filled with the contrast agents. The barium sulfate particles 1 and 40 μm in diameter were often larger than the arterial capillaries. Consequently, the lung acted as 'physiological filter' for particles larger than 5 μm. Since Angiofil is not particle-based such phenomena are impossible. The liquid Angiofil, however, caused emboli in the heart chambers, which probably caused the death of the mouse and prevented significant flow via the arteries to other organs. External pumping (perfusion pump) may help to master this problem properly.[9]

It should be noted that Angiofil has further advantages. The viscosity triggers the embolization. It directly determines, which calibers of the vessels are filled or not. Angiofil of high viscosity leads to micro-embolism and to the occlusion of the micro-circulation.[7] Lowering the viscosity by diluting the contrast agent, the oily liquid also penetrates into smaller vessels. In general, one can recommend for micro-angiography ratios between 1:3 and 1:5 for the iodized oil vs. diluent, v/v. If only the main vessels should be visualized, the viscosity can be kept high. If the small vessels and capillaries are of interest, the viscosity should be decreased adding diluent.

3.3 Comparison of Angiofil and Microfil

Alternatively to conventional radio-opaque liquids or particle-based suspensions, casting techniques have commonly been used to stain the vessels post-mortem.[10] Here, blood vessels have been filled with resins, such as methyl methacrylate, that polymerize within a certain period of time. Upon removal of the biological tissue, a solid but fragile 3D network of the vessel morphology can be obtained. Such a construct can be easily imaged taking advantage of tools such as (synchrotron radiation-based) micro CT or confocal microscopy.

Compared to Angiofil, the casting methods including Microfil casting are more difficult and time-consuming, since mixing and polymerization are additional steps to be performed. Even more important, the tissues have to be post-treated and are not available for histology.

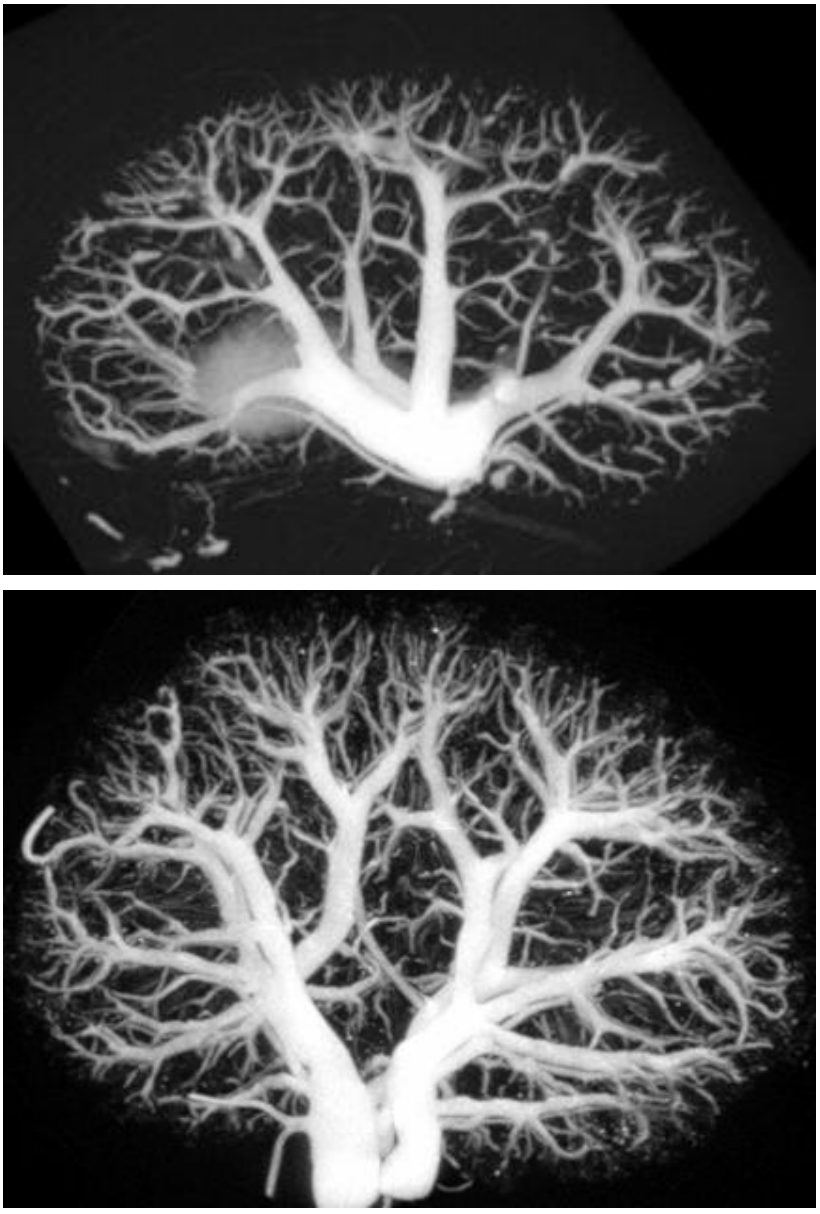

Fig. 4. The maximum intensity projection images were obtained from mouse kidneys stained with Angiofil (top) and casted with Microfil (bottom).

In order to demonstrate that Angiofil is as powerful as Microfil, Fig. 4 shows the direct comparison using mouse kidneys. The two images are maximum projections of the vessel tree. They nicely show the typical morphology of the kidney vascularization.

The maximum intensity projection images show that both procedures can be readily used to visualize the vessel trees of dedicated organs. Microfil, however, results in enhanced image clarity, because the radio-contrast of Microfil is higher than that of Angiofil.

4. CONCLUSION & OUTLOOK

The radio-contrast agent Angiofil provides images of the vascular system in rodents with micrometer resolution that are comparable to established staining and casting procedures but do have significant advantages especially in the handling and time efficiency. The images obtained show that Angiofil provides contrast in the vascular system without the time-consuming sample preparation steps.

Angiofil is a transparent orange-colored liquid, which is not ideal for blood vessel labeling. A rather dark and opaque appearance would permit the user to directly see the perfusion near the organ's surface. This could be achieved adding Sudan's Black or Cibacron Blue to the Angiofil liquid.

Although Angiofil is restricted to post-mortem applications such as forensic or small animal research, it is obvious that Angiofil has the potential to replace casting agents or particle-based contrast agents. Casts, however, have certain advantages, because they can be subjected to scanning electron microscopy analysis, which principally allows for imaging down to nanometer scale. Nevertheless, casting often fails to maintain the entire vascular network including capillaries. The cast specimens are typically prone to damage during transport. Finally, casting is a time-consuming process that involves the critical polymerization. Here, perfusion of individual organs with Angiofil or injecting the contrast agent into the animal is straightforward. If the radio-contrast of Angiofil can be significantly improved, it will replace castings in many applications.

In conclusion, Angiofil compares well to established compounds while offering clear benefits. There is no need of time-consuming sample preparation. Upon micro CT scanning, the organs are readily available for further comparative histological analysis. The user can tailor the viscosity of the contrast agent and thus trigger caliber-dependent visualization of the vessels. Angiofil is a pure liquid, thus no sedimentation or filtering effects by body tissue is observed. Reengineering Angiofil in order to achieve an even increased radio-contrast is desirable, since this property currently prevents being the clear alternative to casting.

ACKNOWLEDGEMENTS

The authors kindly acknowledge the support of Richard Dirnhofer and Erich Gygax, who largely provided infrastructure and personal commitment, Heinz Lüscher for constant support and a stimulating environment and Felix Beckmann and Julia Herzen for the support during the data acquisition at HASYLAB/DESY. Financial support is greatly acknowledged from Forim-X, Fumedica, Virtopsy Foundation, Universities of Lausanne, Bern, Fribourg, and from the Swiss Federal Institute of Technology. The HASYLAB kindly provided beamtime in the frame of an approved proposal.

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