

FIG. 2. The metabolic activities of P450 enzymes in the liver microsomes prepared from PXB mice. The liver microsomes were prepared from RIF- or vehicle-treated PXB mice. The activities of P450 enzymes, CYP1A2 (A), CYP2B6 (B), CYP2C8 (C and D), CYP2C9 (E and F), CYP2C19 (G), CYP2D6 (H), and CYP3A4 (I and J), were determined. The experimental conditions are summarized in Table 2. The fold induction in the RIF-treated group compared with vehicle-treated group was calculated. The metabolic activity of each of the mouse liver microsomes was determined from the means of duplicate assay. Each bar represents the mean \pm S.D. of three mice. Statistically significant from the vehicle-treated group; *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.

The mRNA Expression of Human P450 Enzymes and Transporters. The mRNA expression of the seven human P450 enzymes, UGT1A1, and transporters, including MDR1 and MRP2, was evaluated in the livers of the PXB mice treated with RIF. The fold induction of mRNA expression of enzymes and transporters in the RIF-treated group is shown in Fig. 3. The magnitude of CYP3A4 induction was the largest among the P450 enzymes, followed by CYP2C8 and CYP2B6. Although the enzyme activities of CYP2C9 and CYP2C19 were increased by RIF treatment, the increase in mRNA expression

was too slight to detect significant difference. No changes in the mRNA expression of CYP1A2 and CYP2D6 were observed. RIF treatment significantly increased the mRNA expression of UGT1A1, but not MDR1 and MRP2.

Discussion

In the present study, in vivo study using PXB mice, we simultaneously investigated the inductive effect of RIF on CYP3A4 and CYP2C enzymes. We demonstrated that concomitant use of RIF

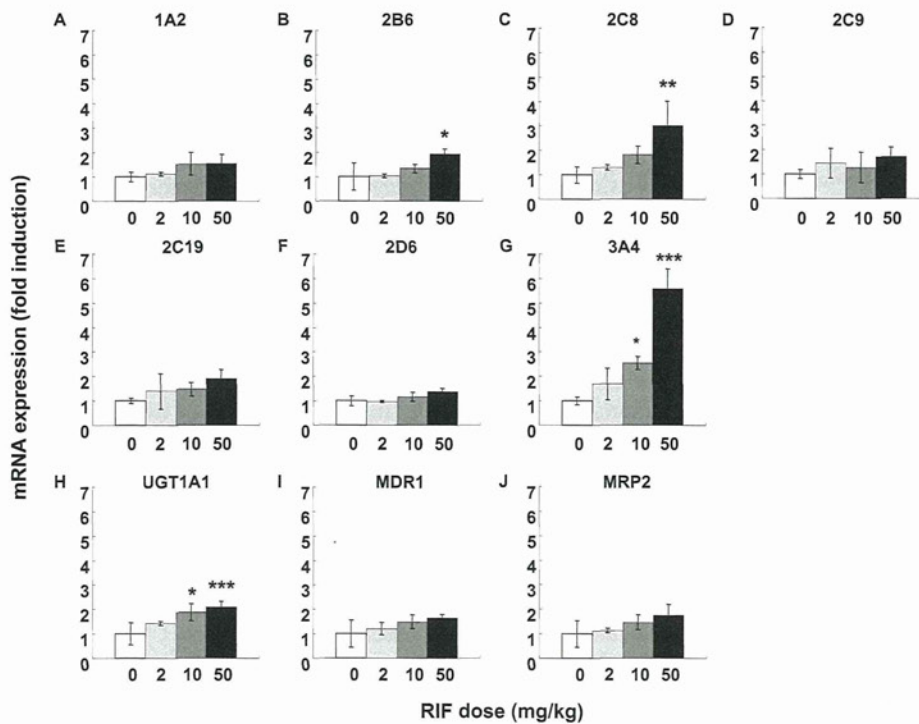


FIG. 3. The mRNA expression levels of P450 enzymes and drug transporters in the liver of PXB mice. The mRNA expression levels of CYP1A2 (A), CYP2B6 (B), CYP2C8 (C), CYP2C9 (D), CYP2C19 (E), CYP2D6 (F), CYP3A4 (G), UGT1A1 (H), MDR1 (I), and MRP2 (J) were determined by SYBR-PCR. The mRNA expression level of each gene was normalized to that of GAPDH. The fold induction in the RIF-treated group compared with vehicle-treated group was calculated. Each bar represents the mean \pm S.D. of three mice. Statistically significant from the vehicle-treated group; *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.

affects the pharmacokinetics of both CYP3A4 and CYP2C substrate drugs and that the inductive effect of RIF on CYP3A4 is greater than that on CYP2C enzymes. In addition, *in vitro* studies using the liver samples after RIF treatment were also carried out to examine the enzyme activities of human P450s and the mRNA expression levels of human P450s, UGT, and transporters. The induction by RIF was observed in the genes whose expression levels were known to be regulated by the human PXR, but no change was observed in the genes not regulated by the PXR (CYP2D6).

To compare the pharmacokinetic data between studies conducted in humans and this study, we selected substrate drugs that have previously been reported to have DDIs with RIF in humans. In a clinical study, the AUC decreases in the P450 substrate drug with concomitant use of RIF (600 mg daily) were 46 to 78% (ROS), 57 to 85% (WAR), and 95% (TRZ) (Villikka et al., 1997; Niemi et al., 2003, 2004; Park et al., 2004). The DDI information on CYP2C19 substrate drugs is very limited, and we could only find information about the urinary excretion data for 4'-hydroxymephenytoin, the main metabolite of MEP (Zhou et al., 1990; Feng et al., 1998). The concomitant use of RIF (600 mg daily) increased the urinary excretion of 4'-hydroxymephenytoin from 1.4- to 2.8-fold (Zhou et al., 1990). Assuming that the urinary excretion amount of the metabolite reflects the metabolic clearance of MEP, the decrease in the AUC would be between 29 to 64% as a result of RIF treatment. These reported clinical data suggest that CYP3A4 is the most susceptible to induction by RIF treatment and that the magnitude of induction of CYP2C8, CYP2C9, and CYP2C19 by RIF seems to be relatively weak compared with CYP3A4. In this study using PXB mice, RIF treatment resulted in the largest AUC decrease in TRZ, followed by ROS and MEP (Fig. 1; Table 3). The response to RIF treatment observed in the PXB mice is therefore similar to that in humans.

It was unexpected that the exposure to WAR was not affected by RIF treatment, despite CYP2C9 induction in the liver microsomes (Figs. 1C and 2, E and F; Table 3). The elimination pathways of WAR other than metabolism by CYP2C9 might have made it harder to detect CYP2C9 induction in the *in vivo* study. In humans, WAR is mainly metabolized to the 7-hydroxyl metabolite by CYP2C9, although WAR is also metabolized to other hydroxyl metabolites by other P450 isoforms (Inoue et al., 2009). Given that the PXB mice used in the present study were derived from the hepatocytes of a single donor, the contribution of CYP2C9 to WAR metabolism in these PXB mice might not be uniformly typical of humans in general. The availability of murine P450 isoforms remaining in the liver of PXB mice could also potentially have affected the overall metabolism of WAR in the *in vivo* study.

We measured the metabolic activities of WAR in the liver microsomes of SCID (severe combined immunodeficiency) mice (the background strain of the PXB mice) and in pooled human liver microsomes to compare them with PXB mice (supplemental figure). The three types of hydroxyl metabolites of WAR, including 7-hydroxy-WAR, were detected in all of the microsomes. However, the 7-hydroxylation activity in the liver microsomes of PXB mice was only one fifth of that in the human liver microsomes. Therefore, the contribution of CYP2C9 to WAR metabolism in the PXB mice may have been smaller than expected. In fact, the formation of another hydroxyl WAR (M2) in the liver microsomes of SCID mice was greater than that in the human liver microsomes, although the absolute metabolic clearance was not determined (supplemental figure). The metabolic activity of murine P450s remaining in the liver could possibly make it difficult to examine CYP2C9 induction by examining the pharmacokinetics of WAR.

To investigate whether a genetic polymorphism could explain the lower CYP2C9 activity in PXB mice, the CYP2C9 genomic polymorphism was determined by Invader assay (BML, Inc., Tokyo, Tokyo) for the cryopreserved human hepatocytes (lot BD85) used in this study. Although no variant sequence was detected in the CYP2C9 gene (data not shown), real-time quantitative reverse transcription-PCR analysis revealed that the mRNA expression level of CYP2C9 in the hepatocytes was relatively low compared with that in other donor hepatocytes (data not shown). In fact, the plasma elimination of WAR in this study seems to be slower than that in the previous study using PXB mice transplanted with a different lot of human hepatocytes (Inoue et al., 2008). Therefore, the main reason for the failure to detect of CYP2C9 induction in the *in vivo* study was probably the low hepatic expression of CYP2C9 in the PXB mice used in this study.

In humans, a therapeutic dose of RIF (600 mg) resulted in an $AUC_{0-\infty}$ of 22,400 to 35,300 ng · h/ml (Polk et al., 2001), which was a similar range of plasma exposure as the PXB mouse receiving the 10 mg/kg RIF (M. Kakuni, unpublished data). Considering the AUC decrease of the substrate drugs in PXB mice and humans caused by RIF treatment, the inductive response in PXB mice seems to be relatively weaker than that in humans. It has been reported that CYP3A4, CYP2C8, CYP2C9, and CYP2C19 are expressed in the human intestine (Kolars et al., 1992; Laple et al., 2003; van de Kerkhof et al., 2008). In addition, it is well known that drug metabolism by intestinal CYP3A4 affects the pharmacokinetics of orally administered drugs (Kato et al., 2003). RIF was previously reported to induce CYP3A4 not only in the liver but also in the intestine in humans (Kolars et al., 1992; van de Kerkhof et al., 2008). Therefore, the decrease in the AUC by concomitant use of RIF in the clinic is accounted for by induction of P450 enzymes both in the liver and in the intestine. In PXB mice, only the liver, but not the intestine, is humanized. Therefore, the intestinal P450 enzymes in PXB mice cannot be induced by RIF, which is a specific human PXR ligand. As a result of the lack of induction in the intestinal P450 enzymes in PXB mice, the reduction of the AUC in the PXB mice would be predicted to be smaller than that in humans. In addition, the hepatic exposure of PXB mice to RIF might be smaller than the expected level, because RIF was administered intraperitoneally, not orally, to PXB mice in this study. The relatively low exposure of the liver to RIF may have result in a weaker induction in the PXB mice.

The induction of CYP3A4 and CYP2C8, CYP2C9, and CYP2C19 by RIF in PXB mice was also demonstrated by examining the enzyme activities using typical substrates for each P450 isoform (Fig. 2). The induction of CYP2B6 in the liver microsomes was also detected (Fig. 2B). This result is consistent with the fact that the expression of CYP2B6 is regulated by the human PXR (Sinz et al., 2008). Next, we determined the mRNA expression levels of other genes, including UGT1A1, MDR1, and MRP2, whose expression levels are also under the regulation of the human PXR (Fig. 3) (Nakata et al., 2006). It was previously demonstrated that RIF led to a small increase in the mRNA expression of these genes using human hepatocytes (Nishimura et al., 2008a,b). In this study, the mRNA expression levels seemed to be slightly increased by RIF in a dose-dependent manner. Statistically significant increase was observed in the mRNA expression levels of UGT1A1 but not in those of MDR1 or MRP2 expression (Fig. 3, H–J). These results might be also attributed to the use of single donor hepatocytes as discussed above.

In the present study, we have performed a DDI study focusing on the human PXR-related induction of CYP3A4 and CYP2C enzymes simultaneously by using the cassette dosing of substrate drugs in PXB mice. We have demonstrated that the PXB mice show a similar response to humans in terms of human PXR-related P450 induction by

RIF. Because the PXB mice used in the present study were derived from the hepatocytes of a single donor, further studies are needed to generalize the present findings by performing DDI studies using PXB mice derived from the different hepatocyte donors.

Considering the magnitude of induction and its contribution to the drug metabolism in clinical situations, CYP3A4 is the most important enzyme to examine during the preclinical development of a new drug candidate. Several groups have established PXR and/or CYP3A4 humanized mice using gene knockout and transgenic techniques (Xie et al., 2000; Ma et al., 2007; Kim et al., 2008; Scheer et al., 2008; Hasegawa et al., 2011). On the other hand, the previous and present DDI studies have demonstrated that the induction of CYP2C enzymes also has a large impact on the pharmacokinetics of CYP2C substrate drugs (Niemi et al., 2003). At present, chimeric mice with a humanized liver, including the PXB mice, are only animal model available to investigate DDIs caused by the induction of CYP2C together with CYP3A4. Furthermore, several groups have reported that the drug-metabolizing profiles in PXB mice are similar to those in humans (Kamimura et al., 2010). Therefore, PXB mice seem to be a suitable animal model to examine the enzyme induction by a drug and its metabolite(s) if these are ligands for the human PXR. In conclusion, PXB mice will provide the opportunity to examine potential DDIs caused by PXR-related enzyme induction in a situation similar to that observed in humans.

Acknowledgments

We thank Tatsuya Matsumi and Dr. Saburo Sugai for supporting this research and reviewing the manuscript.

Authorship Contributions

Participated in research design: Hasegawa and Tahara.

Conducted experiments: Hasegawa, Tahara, Inoue, Kakuni, and Tateno.

Performed data analysis: Hasegawa.

Wrote or contributed to the writing of the manuscript: Hasegawa, Tahara, and Ushiki.

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Fixation methods for electron microscopy of human and other liver

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Received: December 29, 2009 Revised: January 25, 2010

Accepted: February 1, 2010

Published online: June 21, 2010

Abstract

For an electron microscopic study of the liver, expertise and complicated, time-consuming processing of hepatic tissues and cells is needed. The interpretation of electron microscopy (EM) images requires knowledge of the liver fine structure and experience with the numerous artifacts in fixation, embedding, sectioning,

contrast staining and microscopic imaging. Hence, the aim of this paper is to present a detailed summary of different methods for the preparation of hepatic cells and tissue, for the purpose of preserving long-standing expertise and to encourage new investigators and clinicians to include EM studies of liver cells and tissue in their projects.

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Key words: Liver; Tissue fixation; Perfusion; Electron microscopy; Biopsy

Peer reviewer: Dr. Lisardo Boscá, Professor, Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain

Wisse E, Braet F, Duimel H, Vreuls C, Koek G, Olde Damink SWM, van den Broek MAJ, De Geest B, Dejong CHC, Tateno C, Frederik P. Fixation methods for electron microscopy of human and other liver. *World J Gastroenterol* 2010; 16(23): 2851-2866 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i23/2851.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i23.2851>

INTRODUCTION

For an electron microscopic study of the liver, expertise and complicated, time-consuming processing of hepatic cells and tissues is needed. The interpretation of electron microscopy (EM) images requires knowledge of the liver fine structure and experience with the numerous artifacts in fixation, embedding, sectioning, contrast staining and microscopic imaging. In addition, the considerable investment in equipment and the costs of personnel, together with the construction and maintenance of dedicated laboratory space and accessory equipment, makes EM investigation of cells and tissues one of the most cost-intensive approaches in biomedical research. The choice of the

right preparation method and the use of good criteria to evaluate the quality of the preparation, together with the right interpretation of the results, is crucial not only for getting the best out of the method, but also to avoid misuse of tax payers money. With sadness, we conclude that the old trade of traditional EM studies is disappearing, and is being partly replaced by immunological techniques and confocal or fluorescence microscopy^[1,2]. Hence, the aim of this special paper is to present a detailed summary of different methods for the preparation of hepatic cells and tissue, for the sake of preserving long-standing expertise and to encourage new investigators and clinicians to include EM studies of liver cells and tissue in their projects. In a recent review and guidelines on the clinical use of liver needle biopsies by Rockey *et al.*^[3], it is stated that, at present, there is only a minor or even negligible role for the use of EM in the diagnosis, prognosis and management of patients with liver disease.

METHODS OF FIXATION AND PREPARATION

Fixation has several goals: (1) to stop metabolism; (2) to fix structures of organelles and molecules in their current position; and (3) to make material accessible and stable during further processing. When metabolic activity in tissues is not arrested, autolytic changes will develop within minutes or even seconds. The need to use extremely fresh tissue, preferably without touching and deformation is therefore of utmost importance. The fixation is also responsible for crosslinking and arresting many molecules with the result that cells, organelles and their substructures are preserved. After embedding, sectioning and contrast staining, these structures become visualized in the electron microscope at magnifications in the range of 50 to 1 000 000 times. The protocols given hereafter can be used for light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

In a situation where experimental animals are used, the method of perfusing a fixative through the portal vein is by far the best method to choose^[4-7]. When a complete liver is not available, injecting the fixative into a piece of tissue not smaller than 1 cm provides comparable results to portal vein perfusion. This method can be applied to experimental or clinical wedge biopsies^[8-13]. An advantage of this method is the fact that it only requires a small opening of the peritoneal wall to collect a small piece of tissue. With regard to needle biopsies, there have been attempts to inject the fixative into the tissue of the biopsy, with quite good results^[8,10,12,14-16]. Unfortunately, these attempts have not resulted in routine applications and immersion fixation remained the standard method of fixing liver needle biopsy specimens. Vascular perfusion through a partly resected liver not used for transplantation, has also been reported to be successful^[17]. In order to complete the series of protocols, we have added a short description of fixation of liver cell cultures.

Important elements in fixation are the chemical com-

position of fluids and the physical conditions during their application. Fluids involved in the fixation process should be isotonic to the cells; in other words, the fixation fluid should be as physiological as possible, with the exception of the fixing compound^[18]. To avoid temperature shock of cells and tissues before the actual fixation sets in, isothermic fluids should be used in principle. The major condition for fixation is to transfer cells and tissues from their natural environment to the fixing environment, as rapidly as possible: this should take not more than a few seconds, be it in the operating room or in the laboratory. Living cells and tissues should not be left alone, not even for a few seconds, when this can be avoided. One should avoid subjecting the cells or tissue to drying or dry conditions, keep them wet and well-immersed in fluid, and watch the meniscus carefully to ensure that it does not touch the cells, because it will destroy them.

Fixation should be applied at the cellular level, i.e. bring all cells instantaneously and simultaneously into contact with the fixative. Immersion fixation of small pieces of tissue by plunging them into a fixative has to be avoided as much as possible. The slow penetration of a fixative into a 1-mm piece of tissue results in bad fixation in the center of the block. During the penetration of the fixative, its constituents react differently with cellular components, thereby changing the composition of the fixative on its way to the center of the block. The vehicle (buffer) is supposed to penetrate more rapidly, at the same time slowing down the fixing compound. Immersion fixation might leave the center of a tissue block unfixed for the major part of an hour. Nevertheless, immersion-fixed liver biopsies might provide sufficient information on the ultrastructure of parenchymal cells, but unfortunately sinusoids and sinusoidal cells are destroyed by immersion fixation.

Perfusion protocols allow a regimen of different fluids to be flushed through the liver^[18]. Since osmium is an expensive and very toxic substance, it is not recommended to apply this fixative by perfusion. We have tried osmium perfusion^[4], but the results were poor^[18]. For post-fixation, the tissue should be cut into pieces that are washed in buffer to avoid chemical reactions between glutaraldehyde and osmium. The tissue blocks are subsequently immersed in the buffered osmium tetroxide. By doing so, one has control over a toxic procedure, while there is free admission of osmium to the cells through open veins and sinusoids, and the rapid application of a second fixative with a different reaction mechanism^[19]. Although ethanol is used to dehydrate the tissue, its capacity to precipitate proteins and harden the tissue contributes to the fixation process. In order to better preserve membranes and their contrast, several additives to the fixatives have been proposed and applied, such as Ca²⁺ or Li⁺ ions, ferrocyanide^[20], tannic acid, and picric acid^[19,21].

Cryotechniques, molecular identification of cellular components, and their three-dimensional reconstruction or cryofracture as an alternative method to chemical fixation are not the subject of this review.

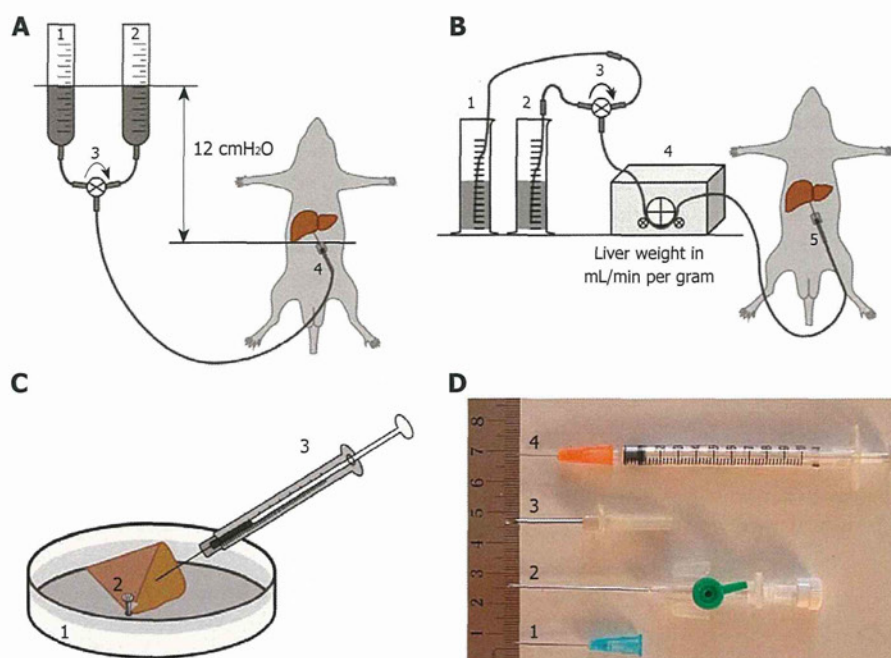


Figure 1 Diagram schematically depicting the different and most frequently used perfusion-fixation methods, including perfusion-fixation needles, to fix hepatic tissue. A: Gravity-mediated perfusion fixation using 12 cm water pressure: (1) pre-perfusion buffer; (2) fixative solution; (3) tri-valve system (e.g. Discofix® C, B. Braun, Switzerland) that facilitates the change-over between both solutions; and (4) cannulation of the portal vein; B: Roller pump-mediated perfusion fixation at a flow of 1 mL/g liver tissue: (1) pre-perfusion buffer; (2) fixative solution; (3) tri-valve system; (4) low-flow peristaltic pump; and (5) cannulation of the portal vein; C: Injection or puncture perfusion fixation, using a needle to inject fixative into a wedge biopsy of about 1 cm × 1 cm × 1 cm dimensions. Injection to approach a rate of 1 mL/min: (1) Petri dish filled with 37°C physiological saline solution; (2) liver tissue wedge fixed by the tip of a forceps; and (3) syringe containing fixative solution; D: Different types of needles and their variants commonly used for portal vein insertion: (1) 18-23 G needle typically used for gravity-mediated perfusion for rat and mouse livers; (2) 18 G needle catheters are recommended for peristaltic-pump-mediated perfusion; (3) 12-13 G needles are typically used for larger animals such as rabbits; and (4) 25 G syringes are ideal for the injection of fixative solution in hepatic tissue. In case of accidental failure of procedure A or B, method C (injection perfusion) can be used to save the preparation.

FIXATION PROTOCOLS

Perfusion fixation of total liver *in situ* (mostly applied to experimental animals)^[5,6,22,23]

The procedure for perfusion fixation of total liver *in situ* is as follows: (1) Anesthetize the animal, preferably with 4.5 mg/100 g body weight Nembutal (which also relaxes the musculature); (2) Fix the animal to a waterproof surgical support with its back down; (3) Shave and disinfect the animal's abdomen; (4) Open the abdominal cavity along the linea alba, with lateral cuts along the ribs and lower segment; (5) Gently move the intestines aside and cover them with surgical cotton or bandages wetted with warm physiological saline to keep them moist and warm (one could also use an infrared lamp); (6) Expose the portal vein and prepare separate, double ligatures around it; (7) Take care that the hepatic artery is included in the ligature; (8) Introduce the largest possible (just fitting) needle into the portal vein, after connecting it to a silicon tube that is connected to the perfusion system (fluids on room temperature, glass vessels or peristaltic pump, Figure 1A and B); (9) Constrict the ligatures independently, taking care not to puncture the portal vein, and make sure to close to the hepatic artery; (10) Start perfusion with glutaraldehyde solution by opening a valve or switching on the peristaltic pump; the flow rate in mL/min should be more or less equal the total weight of the liver in grams; (11) Incise the vena cava to allow fluids to escape from the vascular

system, and aspirate fluids when necessary; (12) Watch the liver change in color and consistency. This process should start within the first minute (typically 15-30 s); (13) Stop the perfusion after 5 min; (14) Gently remove the liver or one or two well-perfused lobes and put them into a Petri dish that contains fixative. Well-perfused liver lobes change their color from dark red to yellow/brown, whereas the consistency changes from soft to hard like a boiled egg. Badly perfused parts of the liver that are entirely or partly soft and still dark red-brown in color should not be processed. Note that some lobes, e.g. the caudate lobe, often show better perfusion than other ones; (15) Clean a razor blade with ethanol and paper tissue (to remove protecting grease) and gently cut 1-mm slices of liver tissue. Do not put any pressure on the tissue while cutting, and make sawing movements with the razor blade; (16) Keep tissue slices covered with glutaraldehyde and cut multiple 1 mm × 1 mm strips under fluid; (17) Cut several strips simultaneously into 1 mm × 1 mm × 1 mm blocks for TEM, or 1 mm × 1 mm × 5 mm strips for SEM and/or 5 mm × 5 mm × 1 mm slices for light microscopy (LM) flat embedding; (18) Total time in glutaraldehyde should not be longer than 20 min; (19) Transfer blocks to washing buffer (which is the buffer of the glutaraldehyde fixative) to remove glutaraldehyde before contact with osmium; (20) Transfer blocks to 1% buffered osmium in small glass vessels and close these with a cap; (21) Postfix for 1 h in osmium, keep the small vessels at 4°C, and shake

Table 1 Dehydration and embedding for TEM and SEM

| TEM | | |
|------------------------------------|--------------------------|---------|
| Ethanol 70% | 15 min | 20°C |
| Ethanol 90% | 15 min | 20°C |
| Ethanol 100% | 30 min | 20°C |
| Ethanol 100% | 30 min | 20°C |
| Ethanol 100%/Epon 1/1 | Overnight | 20°C |
| Epon | 1 h | 20°C |
| Epon 1 (overnight) | BEEM capsules on top | 20°C |
| Epon | Fill BEEM capsules | 20°C |
| Oven | 67 h | 40-60°C |
| SEM | | |
| Ethanol 70% | 15 min | 20°C |
| Ethanol 90% | 15 min | 20°C |
| Ethanol 100% | 30 min | 20°C |
| Ethanol 100% | 30 min | 20°C |
| Ethanol 100% | Overnight | 20°C |
| Freeze and fracture | Liquid nitrogen | -196°C |
| Ethanol 100% | 30 min | 20°C |
| Critical point drying, phase 1 | CO ₂ /ethanol | 16°C |
| Phase 2 or HDMS drying (see below) | 80 bar | 35°C |
| Sputter coating | Pt/Au | |

Cutting of ultrathin sections on an ultramicrotome, contrast staining with lead and uranyl salts. TEM: Transmission electron microscopy; SEM: Scanning electron microscopy.

the fluid gently and regularly; (22) Transfer the tissue blocks to the second washing buffer (osmium-vehicle buffer), and wash once or twice to remove osmium; (23) Transfer the blocks to 70% ethanol (v/v); (24) Change 70% ethanol three times; and (25) Transport in 70% ethanol or follow the protocol for further dehydration in ethanol and embedding or critical point drying (see common trunk) (Table 1).

For a summary of necessary chemicals and solutions, composition of fluids, see Table 2.

Fixation of liver wedge biopsies (mostly applied to human liver tissue) (Figure 1C)

Wedge biopsies of roughly 1 cm × 1 cm × 1 cm (or less), including the Glisson's capsule at two sides of the wedge, are taken from the margin of a liver lobe as soon as the surgeon/operator has access to the liver. The operator should be cooperative in order to assure that freshness of the tissue is an absolute priority. Directly after opening of the abdomen *via* a chevron incision, the liver is visualized and a wedge is taken with a sharp scissor from the edge of segment 5 or 6, without manipulating the tissue. Bleeding at the biopsy site can easily be controlled using diathermia or argon laser. In a consecutive series of 150 patients no rebleeding at biopsy sites was reported (unpublished results). (1) The tissue is immediately transferred to a 50-mL vessel, filled with PBS (pH 7.4) at 37°C; (2) The tissue is transported to an annex to the operation theater, where injection perfusion with glutaraldehyde fixative is performed in a Petri dish filled with saline; (3) The wedge biopsy is held at a corner by a forceps and is injected by a 25 G syringe from multiple sides with 1.5% glutaraldehyde fixative, until a discoloration and hardening of the tissue, comparable to total liver perfusion, is obtained; (4)

Table 2 Addendum summarizing chemicals necessary for fixation of liver tissue

| Compound | Quantity |
|---|-------------------|
| Cacodylate buffer 0.2 mol/L, pH 7.4 | 700 mL |
| Sodium cacodylate trihydrate (Sigma, C0250) | 22.9 g |
| MQ-(purified by ion-exchange)-water | 674.8 mL |
| HCl (0.2 mol/L) | 25.2 mL |
| Filter through 0.2 µm Millipore or Wattman filter paper | |
| Cacodylate buffer 0.15 mol/L, pH 7.4 | |
| Cacodylate buffer 0.2 mol/L | 900 mL |
| MQ-water | 300 mL |
| Filter through 0.2 µm Millipore or Wattman filter paper | |
| Glutaraldehyde fixative, 1.5% in cacodylate buffer 0.067 mol/L | |
| Cacodylate buffer 0.2 mol/L | 575 mL |
| Sucrose (Sigma, S0389) | 17.215 g |
| Glutaraldehyde solution 70% (Sigma, G7776) | 36.96 mL |
| MQ-water | 1113.04 mL |
| Filter | |
| Osmium tetroxide 1% in phosphate buffer ¹ | 90 mL (6 × 15 mL) |
| Osmium tetroxide 4% for EM (Sigma, 75632) | 22.5 mL |
| Phosphate buffer 0.2 mol/L | 45 mL |
| MQ-water | 22.5 mL |
| Washing buffer 1 | |
| Cacodylate buffer 0.2 mol/L | 30 mL |
| Sucrose | 0.9 g |
| MQ-water | 60 mL |
| Washing buffer 2 | |
| Phosphate buffer 0.2 mol/L | 45 mL |
| MQ-water | 45 mL |
| Phosphate buffer 0.2 mol/L pH 7.4 (200 mL) | 200 mL |
| 0.2 mol/L Na ₂ HPO ₄ ·2H ₂ O (17.8 g in 500 mL MQ-water) | 162 mL |
| 0.2 mol/L NaH ₂ PO ₄ ·H ₂ O (13.8 g in 500 mL MQ-water) | 38 mL |

Fluids and chemicals (volumes to be adapted to needs). ¹An alternative to the osmium postfixation is addition of 1.5% potassium ferrocyanide to 1% osmium in veronal acetate buffer (pH 7.4) at 4°C for 2 h, which strongly enhances the contrast of membranes in cells and tissues^[20] (see Figure 13).

Care should be taken that the injection needle does not operate as a biopsy needle; it should be and stay completely filled with glutaraldehyde during injection into the tissue; (5) After injecting the needle into the tissue, parallel to the capsule rim, the needle should be withdrawn a few millimeters before starting injection of the fixative fluid. The idea is to create a space within the tissue in which the fixative can spread out; (6) Injections should be repeated several times until the blood is flushed out of the tissue and the consistency has changed to that of a hard-boiled egg; (7) The time between excision of the wedge and perfusion with fixative is critical. Total glutaraldehyde time (before washing and before osmication) should not exceed 20 min; and (8) After perfusion with glutaraldehyde, the biopsy is rapidly transported to the laboratory, where slices (1 mm thick), strips (1 mm × 1 mm × 5 mm), small flat slices (5 mm × 5 mm × 1 mm) and tissue blocks (1 mm × 1 mm × 1 mm) can be cut under fluid with a clean, sharp razor blade.

Immersion fixation of liver needle biopsies (Figure 1D)

The percutaneous liver biopsy procedure starts with local anesthesia with 1% lidocaine. Under real-time ultrasound guidance, the liver and the biopsy needle are imaged throughout the procedure. The biopsy is taken by a cut-

ting-type needle and a spring-loaded device. A 16-gauge needle is placed into the liver parenchyma before the device is triggered. This results in a liver specimen of 1-2 cm in length, with the diameter being dependent on the size of the biopsy needle (1-1.8 mm). After collecting the biopsy, the fresh tissue is placed in a Petri dish in which 3 mm × 1 mm × 1 mm pieces of the ends of the cylinder are cut. The remaining tissue is used for diagnostic purposes in the pathology department and separately undergoes routine pathology fixation. This procedure should not take longer than 30 s. The two cylinder-shaped tissue pieces of 3 mm × 1 mm are covered with fixative, cut into 1 mm × 1 mm × 1 mm pieces and transferred to a 15-mL vessel that is filled with 1.5% glutaraldehyde fixative at room temperature. The vessel is gently shaken a few times. The total glutaraldehyde time (before washing and osmication) should be no less than 1 h, in order to allow the fixative to penetrate the tissue. After glutaraldehyde, the tissue will be further processed in washing buffer, osmium fixative, and ethanol dehydration.

Fixation of liver cell cultures

The procedure is as follows: (1) cells are cultured on 1 cm diameter glass or plastic coverslips (0.17 mm thick) in wells of culture dishes; (2) cultures are washed three times with 37°C buffer to rinse away dead cells, cellular debris and precipitates; (3) culture medium is replaced with glutaraldehyde fixative; (4) one should take care that the cells are always covered by a layer of fluid, so that the meniscus does not touch the cells; (5) fluids are changed several times to ensure complete replacement; (6) wash in cacodylate buffer; (7) postfix in 1% osmium tetroxide in phosphate buffer; (8) wash in phosphate buffer; and (9) dehydrate by starting with 70% ethanol (Table 1, Figures 2 and 3).

Critical point drying (CPD) is used routinely to dry samples for SEM^[24]. Nation^[25] has reported an alternative method that consists of air-drying of insect tissues by rapid evaporation of hexamethyldisilazane (HMDS), which has a very low surface tension. This is supposed to avoid fracturing and destruction of the specimen during drying. In spite of the advantages of HMDS, such as the absence of high pressure equipment, CPD seems to be the most frequently used method. Drying of biological samples by CPD also causes a shrinkage of about 30% of the volume^[26]. We have routinely and successfully applied HMDS to cultured liver cells^[27]. HMDS drying takes only 3 min instead of the laborious and time-consuming CPD process. We also have applied HMDS drying to perfusion-fixed liver tissue, and during a comparison with CPD, no differences could be seen between the two methods (Figure 3).

After 100% ethanol, samples are immersed for 3 min (cell cultures) or 10 min (liver tissue) in 100% HMDS. After HMDS, the samples are removed from the culture wells and excess HMDS is blotted away by filter paper. The samples are then transferred to a desiccator for 25 min to avoid water contamination. After drying, the samples are mounted on stubs (Figures 4 and 5) and are

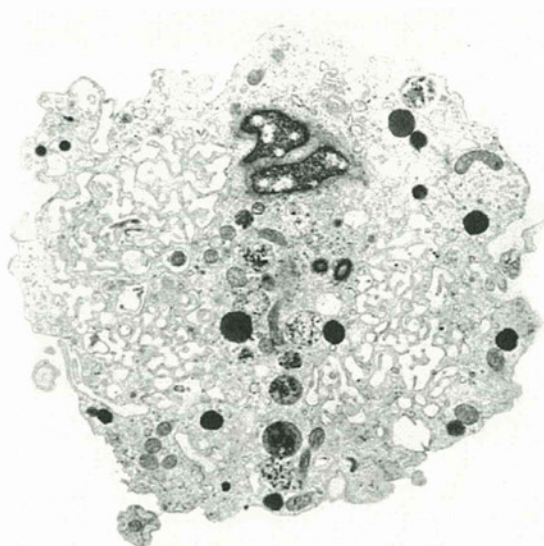


Figure 2 Transmission electron microscopy (TEM) image of an ultrathin section of a plastic-embedded liver sinusoidal endothelial cell after isolation. In the upper part of the picture is a small portion of the nucleus. The labyrinth-like structures in the cytoplasm represent fenestrae that are internalized during the isolation procedure. Electron-dense granules represent lysosomes that reflect the high digestive capacity of these cells. Magnification 6300 ×.

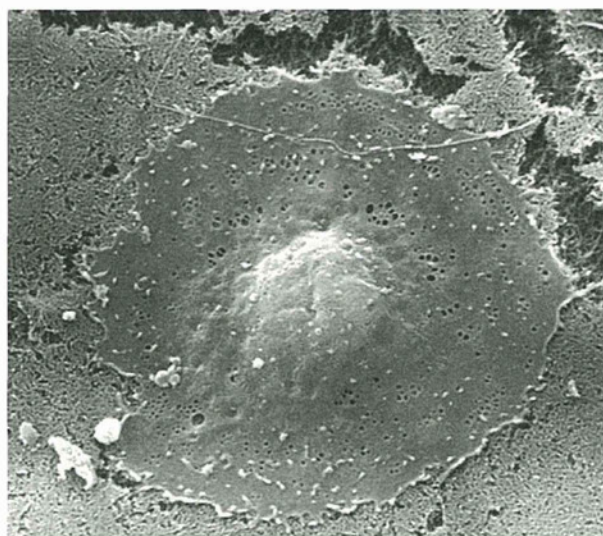


Figure 3 Scanning electron microscopy (SEM) image of a critical-point-dried liver sinusoidal endothelial cell in culture, 2500 ×. After isolation and purification, cells were seeded on a layer of collagen. Besides the centrally located, bulging nucleus, fenestrae can be easily observed in the surrounding thin, flat cytoplasm.

sputter-coated with 10 nm gold and made available for SEM investigation.

Preparing sections for LM

Follow the instructions given for TEM and make sections of 0.5-1 μm, stain with Toluidine Blue (1% with 2% borate in distilled water), cover sections with Entellan and a 0.17-μm glass coverslip. In case of sufficient contrast, use bright field microscopy, in case of low or insufficient contrast, use phase contrast or interference contrast.

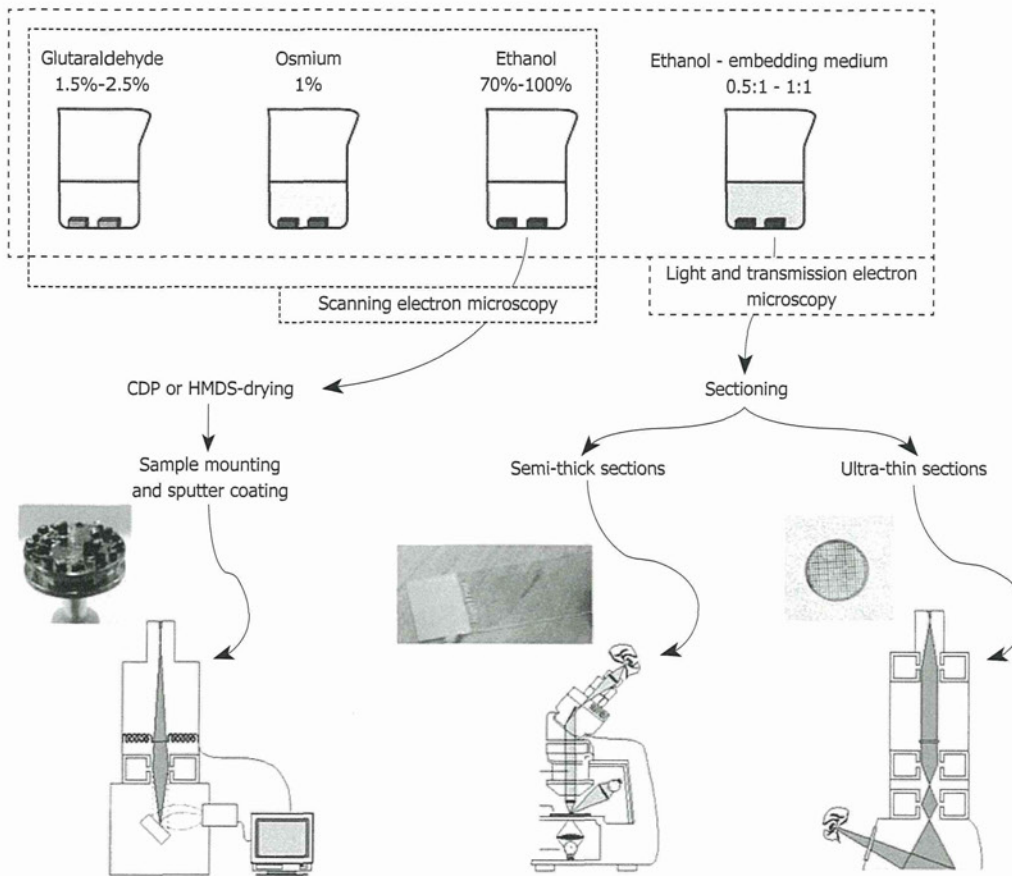


Figure 4 Summary of the different steps in the preparation of liver tissue after aldehyde fixation (top figure) and the subsequent tissue sample preparation steps for the different modes of microscopy (bottom figure). Good laboratory practice should involve all three visualization modes in order to collect fine structural and topological data, together with insights into the interior of the sample. Left: SEM; Middle: Light microscopy (LM); Right: TEM.

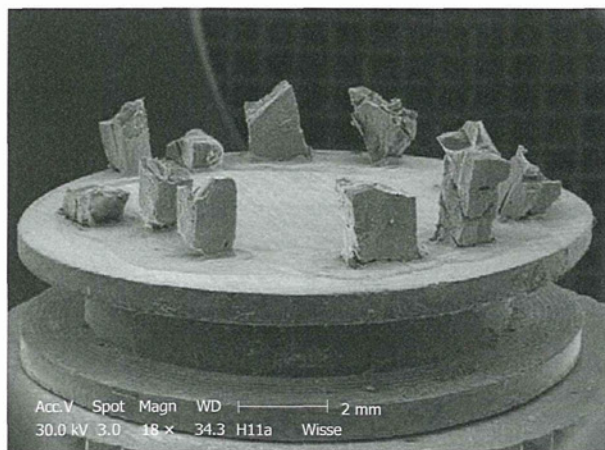


Figure 5 Typical stub of aluminum (diameter 1 cm) bearing 11 pieces of liver tissue of an injection-fixed human liver wedge biopsy observed by SEM at low magnification. Strips of tissue in 100% ethanol are frozen at -196°C, fractured, critical point dried, sputter coated and glued to this carrier. The top surface of the specimens is observed, and imaging is achieved with secondary electrons. This picture is taken by a small CCD camera fitted to the microscope to observe specimens directly.

KEY ASPECTS TO CONSIDER

Anesthesia

When using experimental animals, fixation should be pre-

ceded by anesthesia. Undoubtedly, the success of perfusion also depends on the handling of the animals. Animals should be minimally stressed before and during the operation. Therefore, it is recommended to have the animals about 2 h before the surgical procedure within the operating room to accustom them to the environment and reduce the level of stress. Stress hormones affect the physiology and fine structure of organs. Furthermore, animals in discomfort are not only difficult to manipulate, but the stress hormones might cause vasoconstriction and directly narrow or even block some vascular beds, thereby affecting the outcome of the perfusion procedure and also the fine structure of the cells.

Some researchers use ether as an anesthetic. Although this is a rapid procedure, it should be discouraged as it does not provide any pain relief, nor does it have muscle-relaxant properties. Furthermore, ether anesthesia is difficult to control, and often leads to an overdose, typically to be encountered during the initial stages of the operation, resulting in body temperature drop, shock and even accidental death of the animal. Animals that are in distress and anesthetized with ether are difficult to handle and operate on and chances for a successful liver perfusion are low. As outlined in the protocol section, agents such as pentobarbital and chloral hydrate, preferably combined with pain inhibitors, are the first-choice anesthetics. The



period of anesthesia should be as short as possible^[22]. Anesthesia can be checked by gently touching the cornea of the animal, followed by gently squeezing the paw pads. When no reaction occurs, the animal is ready for operation. When laparotomy is performed, hypovolemia should be prevented and veins and arteries should be well perfused and red^[22].

Preperfusion

The need for preperfusion with buffered physiological saline is still being discussed. The argument used in its favor is to wash plasma and blood cells away in order to avoid coagulation, precipitation of blood constituents and aggregation of blood cells by glutaraldehyde. Such precipitates could impair the flow of fixative through the liver. Theoretically this is correct, but experience has taught us that plasma constituents and blood cells do not have enough time to react and precipitate when glutaraldehyde is flushed through the liver straightaway. It is possible to flush the liver, e.g. of a rat, within 15-30 s without any notable obstruction. After 30 s, the way should be free to continue perfusion of the fixative. The point is that fixative and biological material need time to react, and perfusion might be successfully established before precipitation, coagulation and cellular aggregation occurs. A compromise might be to fill the tubes used for perfusion with buffered physiological saline, which results in a small "wave" of saline preceding the glutaraldehyde, the two being sequentially distributed through the entire microcirculation of the liver^[22].

When preperfusion is still preferred, not more than 30 s preperfusion with buffered physiological saline at a physiological flow rate is necessary to free the liver of plasma and red blood cells. From this moment onwards, glutaraldehyde fixation can be started. The success of perfusion correlates with disappearance of red blood cells from the liver and can be followed by the change in color of the organ (dark red brown to yellow-brown). The presence of blood can also be monitored by observing the effluent. The success of fixation correlates with the change in consistency of the organ (from soft and pliable to rigid/stiff like a hard-boiled egg). Successful perfusion fixation shows the start of these phenomena within 20 to 30 s.

Air bubbles might form within the flexible tubing that connects the perfusion needle with the vessels that contain the perfusion fluids. Bubbles appear often when improperly cleaned tubing is used, or are caused by long waiting times or temperature differences between the different fluids. Air bubbles are disastrous in liver perfusion and act as blockers of the liver microcirculation. They are a major source of perfusion failure^[5]. It is therefore recommended to check the tubing regularly. Air bubble traps are commercially available and are being used in clinical settings. They certainly are advised when using larger animals (e.g. rabbits, dogs or mini pigs) when high-volume perfusion is required.

As soon as perfusion is started, it is mandatory to open the vascular system by incising the vena cava in or-

der to avoid the buildup of pressure. Enhanced pressure damages endothelial cells as well as parenchymal cells^[28].

Formaldehyde has found no application in perfusion fixation of the liver. Glutaraldehyde has better stability in solution and has two reactive groups per molecule, therefore, it cross-links more easily, has lower osmotic pressure and gives better results^[29]. Nevertheless, it might be useful to preperfuse the liver with formaldehyde. Since formaldehyde has a much slower reaction time than glutaraldehyde^[29], it could be possible to preperfuse the liver with 3%-4% buffered (and freshly prepared) formaldehyde. Care should be taken for the high osmotic pressure of formaldehyde solutions. Formaldehyde preperfusion would allow flushing of blood and its constituents from the tissue (within 1 min) and starts mild fixation at the same time. Glutaraldehyde perfusion can be started directly thereafter and makes clear the way to do the real job of fixation in the absence of plasma and blood cells.

The choice of a roller pump *vs* gravity perfusion is a matter of personal preference. The roller pump releases moderate artery-like pulses of fluid (depending on the tube diameter), whereas gravity flow is constant and results in constant flow or pressure. The choice is considered not to be decisive for perfusion quality.

Since the liver circulation can be characterized as a high flow/low pressure system, discussions about perfusion pressure during fixation have limited value. It is believed that flow rates within wide limits do not enhance perfusion pressure, since the open system and sponge-like structure of this typical microcirculation do not allow pressure to build up. This situation changes when the fixative precipitates blood proteins, aggregates blood cells and introduces coagulation. When attention is paid to this condition, physiological flow rates (the rule of thumb: weight of liver in grams = milliliters of fluid per minute) should give good results.

Choice of the perfusion needle

Experience over the years has taught us that the outcome of fixation by perfusion also depends on the choice of the needle. The diameter of the needle, i.e. the gauge (G), determines the maximum flow to be delivered. This gauge value, together with the liver blood flow and pressure for a particular type of species, are important factors to consider. 18 G needles, for example, with a typical diameter of 1.2 mm, have a maximum perfusion flow by 12 cmH₂O gravity of 80 mL/min. This is evidently too low when livers of rabbits (177 mL/min) or dogs (303 mL/min) are concerned^[30].

18-23 G needles (diameter 1.27-0.64 mm) are ideal for liver perfusion in animals such as rats and mice (Figure 1D1); whereas the 12 G (diameter 2.77 mm) needle type is recommended for larger species such as rabbits and minipigs (Figure 1D3). One can benefit from studying a catalogue of the needle catheter-type of needles that are on the market. A needle catheter (Figure 1D2) comprises a metallic needle-guide that consists of a cannula with an end grip - the catheter - that covers the needle-guide when it is retracted after cannulation. Hence, after the needle