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Vital staining of blood vessels and bile ducts with carboxyfluorescein diacetate succinimidyl ester: a novel tool for isolation of cholangiocytes

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Summary. Background and aim: Current methods for visualization of the blood vasculature, biliary tree and for isolation of vital cholangiocytes are afflicted with a plethora of technical difficulties, especially in mice. In this project, we propose a novel, reliable and straightforward alternative technique for histological demonstration of blood- and biliary systems and derivation of vital cholangiocytes.

Methods: Intravital retrograde perfusion of bile ducts was performed in twenty wild type mice. Liver and gallbladder were exposed by median laparotomy. Using a venous catheter, the gallbladder was cannulated, a few millimeters of the liver edge were cropped to allow free outflow of the perfusate, and carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) solution was retrogradely infused. Thereafter, formaldehyde solution was either injected through the same catheter, or the liver was immediately dissociated into a single-cell suspension for FACS-analysis. Intravital perfusion of the vascular system was performed in ten Lewis rats by direct intra-arterial injection of CFDA-SE into the abdominal aorta. The specificity and sensitivity of CFDA-SE labeling was controlled using Indian ink or cytokeratin 19 immunohistochemistry respectively.

Results: Upon histomorphological analysis of cryo- and paraffin sections, strong fluorescence was noted in large and small bile ducts throughout the entire liver and in the vascular system after infusion of the CFDA-SE solution. In preliminary FACS-experiments, we succeeded in separating cholangiocytes based on combined CFDA-SE-staining and cell size.

Conclusions: Visualization of liver architecture and the isolation of cholangiocytes is feasible using a fast and cost-effective method of retrograde perfusion and vital fluorescent labeling of mouse bile duct epithelium and vascular endothelium with CFDA-SE.

Key words: CFDA-SE, Cholangiocytes isolation, Retrograde perfusion, Vessel visualization

Introduction

The interplay between portal hypertension, cholestasis and fibrogenesis remains an object of strong research interest of liver pathologists (Pinzani and Gentilini, 1999; Bosch and Garcia-Pagan, 2000; Iwakiri et al., 2008; Rockey, 2008; Bosch et al., 2010). The observation of morphological and proliferative characteristics of the biliary tree and the liver vasculature is a key element in the understanding of the complex mechanisms and processes of hepatic physiology and pathology. It is crucial for modeling and

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Abbreviations: ASMA, alpha-smooth muscle actin, CFDA-SE, carboxyfluroescein diacetate succinimidyl ester (CFDA-SE) , CK-19, Cytokeratin 19, DMEM, Dulbecco's modified Eagle medium, FACS-analysis. fluorescence activated cell sorting, PBS, Phosphate buffered saline

assessing therapeutic effects, especially in the therapy of liver fibrosis (Masyuk et al., 2001, 2003; Andrade et al., 2006; Scholten et al., 2010; Chu et al., 2011; Nakanuma, 2012). One type of effector cells in these processes are cholangiocytes (Harada et al., 2005), however their isolation from the murine liver is afflicted with a multitude of technical difficulties (Yahagi et al., 1998; Alpini et al., 2003; Nakagome et al., 2007).

The visualization of the 3D-networks of blood- and bile duct capillaries is an important component of the quantitative and qualitative evaluation of liver morphology (Gaudio et al., 1991, 1993; Menger et al., 1991; Onori et al., 2000). Indian ink infusion, corrosion casting and immunohistochemical staining are standard techniques in use for that specific purpose (Fickert et al., 2002; Van Steenkiste et al., 2010). Lectins (Hansen-Smith et al., 1988; Roussel et al., 1992; Dickie et al., 2006), DiI (Nakano et al., 1998; Zhang et al., 2005) as well as specific antibodies e.g. PECAM-1 (CD31) and cytokeratin 19 (CK-19) are widely used to visualize the vascular- or biliary patterns, respectively. Each of these methods has significant advantages but also inherent limitations: the obtained results are highly dependent on tissue processing (for example, Indian ink particles can be washed out by histochemical procedures), tissue fixation (the membrane incorporated DiI is not resistant to lipophilic solvents) (Li et al., 2008), and specificity of binding (some antibodies are hampered if tissues are formalin or glutaraldehyd fixed or paraffin embedded) (Grizzle, 2009; Paavilainen et al., 2010; Schnell et al., 2012). Thus, a new method for vital staining of vascular endothelium and the luminal surface of biliary canaliculi and bile ducts in order to enhace findings from already existing methods may be useful (Hossler and Douglas, 2001; Elvevold et al., 2008). Cell tracing has led to widespread success as an important and often used method in recent years (Horan et al., 1990; Oostendorp et al., 2000; Lulevich et al., 2009; Kircher et al., 2011). In 2008, Li and Song proposed a protocol for direct labeling and visualization of blood vessels in small experimental animals using the lipophilic carbocyanine dye, CM-DiI (Li et al., 2008).

In our study, we were searching for substances applicable to the visualization and histological examination of the biliary and vascular systems. Our special interest was their suitability for the isolation of vital cholangiocytes. We chose CFDA-SE as a prospective candidate for this purpose. This fluorescein based vital dye is widely used as a non-specific cell marker for the determination and quantification of cell division both *in vitro* and *in vivo* (De Clerck et al., 1994; Fazekas de St Groth et al., 1999).

In 1990, for the first time, Weston and Parisch proposed the use of CFDA-SE, often called CFSE, for the labeling of lymphocytes (Weston and Parish, 1990). The substance consists of a fluorescein molecule containing a succinimydyl ester functional group and two acetate moieties. This solution is initially colorless and non-fluorescent. CFDA-SE passively penetrates into the cell. The acetate groups of the molecule are cleaved by intracellular esterases to yield a highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester (CFSE). The succinimidyl ester reacts with the amine groups of intracellular proteins and forms stable covalent fluorescent conjugates (Parish, 1999). CFDA-SE induced fluorescence is suitable for flow cytometry and for fluorescence microscopy (Lyons, 1999). Based on the physiological properties of CFDA-SE, we present, to our knowledge for the first time, an alternative technique for supravital labeling and histological demonstration of the hepatic vascular system and biliary tree, and for the isolation of bile duct epithelial cells.

Materials and methods

Animals

Male and female wild type mice of different strains and ages (3-12 months) with a mean body weight of 35 g were housed in the veterinary care facility of the University of Cologne Medical Center. A total of 15 mice were used in this study for visualization of the biliary tree. 5 mice were used for visualization of blood vasculature.

10 rats of different sex and strain, average weight 250 g were housed in the veterinary care facility of the University of Erlangen Medical Center and used for visualization of blood vessels.

All animals were submitted to a 12 h light–dark cycle and were fed a standard chow diet (Altromin®, Altromin GmbH, Hamburg, Germany) and water ad libitum. German regulations for the care and use of laboratory animals were upheld at all times. Experiments were approved by the governmental animal care and use office (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany, Protocol No. 20.10.171) and were in accordance with the German Animal Welfare Act.

Preparation of working solutions of CFDA-SE for vital staining

A Vybrant® CFDA-SE cell tracer kit was provided by Invitrogen. The stock solution of CFDA-SE was prepared according to the manufacturer's instructions. Solutions of concentrations of 50, 100, 150, 180 and 200 μM were prepared by diluting a stock solution into 37°C warm low glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen/Gibco) and used immediately to perform perfusions.

Retrograde perfusion of the biliary system

The animals were briefly sedated with Isoflurane® (Baxter, Unterschleißheim, Germany) and then killed by cervical dislocation to perform the intravital retrograde perfusion of the bile ducts.

Sacrificed animals were fixed in supine position. Liver and gallbladder were exposed by median incision

from the xiphoid process to the pubic symphysis.

A small dissection forceps was placed in the caudal part of the processus xiphoideus in order to elevate the sternum, improving access to the gall bladder. The liver lobes were repositioned to expose the portal vein and extrahepatic bile duct.

The gallbladder was cannulated using a venous 26G catheter (Becton Dickinson, Sweden). Then, a few millimeters of the liver edge were cropped to allow free outflow of the perfusate and the bile duct (ductus choledochus) was closed using microvascular clamps to prevent outflow of the perfusate into the intestine. Finally, the biliary tree was retrogradely perfused. Based on preliminary experiments carried out to establish the volume of solution required for sufficient elimination of bile, slow injection of 5-7 ml solution containing CFDA-SE for approximately 3-5 minutes was performed. The colour of successfully perfused livers slightly faded in relation comparison to the parenchyma of the non-perfused organ.

After 15 min incubation time, the biliary system was perfused with paraformaldehyde (4%, 3-5 ml). Immediately after perfusion, all lobes of the liver were divided for immersion fixation in 4% paraformaldehyde or cryopreservation in liquid nitrogen. Finally, the liver samples were submitted to histological analysis.

Control Tissues

Two mice in this study were used as controls of autofluorescence in the liver parenchyma. For this purpose, animals were perfused with DMEM or Phosphate buffered saline (PBS)- Dulbecco, without Ca^{2+} and Mg^{2+} (Biochrom AG, Berlin, Germany) for 5 min. The perfusion was then completed by infusion of 3-5 ml 4% formaldehyde freshly prepared from paraformaldehyde solution. All of the following procedural steps were identical to CFDA-SE perfused samples.

Perfusion of the vascular system

To perform the perfusion, the rats were anesthetized with Isoflurane®, a general inhalation anesthetic, and placed on a heating plate surface (38°C). 1.5 % Isoflurane® was administered via a calibrated Isoflurane vaporizer through which a flow of air was passed.

The rats underwent laparotomy, followed by a standardized manipulation to expose the aorta and vena cava as previously described (Polykandriotis et al., 2011).

Control Tissues

To control autofluorescence of the blood vessels, tissue sections from parenchymatous organs, spinal cord and muscles of the anterior and medial femoral regions of two animals were examined. The control animals were perfused with a heparin solution in NaCl 0.9% for 10 minutes. Immediately after the perfusion, samples were collected and either frozen or fixed in 4% paraformaldehyde and embedded in paraffin.

Histology

Samples of liver, muscles, kidney, heart and brain were each divided into two pieces. One piece was embedded in Tissue-Tec® (O.C.T. compound Tissue-Tek®, Sakura Finetek Europe BV, Zoeterwoude, the Netherlands) and then frozen in liquid nitrogen-cooled isopentane.

The frozen tissues were cut into 5, 10 and 20 μm thick sections, mounted on Superfrost® Plus microscope slides (Menzel GmbH, Braunschweig, Germany) and then stored at -80°C.

The other part of the tissue samples was immersion-fixed in 4% formaldehyde for 72 hours and prepared for paraffin embedding, or was sectioned by vibratome. 20, 30 and 50 μm thick free-floating vibratome sections were rinsed in PBS for 30 minutes, mounted on chrome-gelatin coated glass slides and covered with ProLong Gold Reagent (Molecular Probes/Life Technologies, Eugene, Oregon). The paraffin embedded tissues were cut into 5 μm sections, deparaffinised in xylene for 10 minutes, dried and then embedded with ProLong Gold.

The prepared slices were observed using a fluorescent microscope, equipped with a filter set F36-503, F36-525, F36-523 (AHF analysentechnik AG, Tuebingen, Germany, www.ahf.de). Different magnifications and, if necessary, oil immersion were used and the intensity of fluorescence was judged semiquantitatively. The images were taken using a slow scan CCD camera (Spot RT3 Slider, Diagnostic Instruments, Inc., USA).

Control of specificity and sensitivity of CFDA-SE labeling

Indian ink and Microfil perfusions.

To verify the successful distribution of CFDA-SE solution within the tissues, perfusion of bile ducts or blood vessels was continued with injection of Indian ink or yellow Microfil® (MV-122) containing 5% of MV Curing Agent (MV-122; Flow Tech, Carver, MA, USA). Indian ink and Microfil® solutions were prepared as described previously (s.o.) (Arkudas et al., 2009).

Indirect immunofluorescence for cytokeratin 19 (CK-19)

The mouse liver sections or cytospin specimens, dried at room temperature for 30 minutes, were fixed with cold acetone, treated with normal donkey serum blocking buffer and then incubated with a rat monoclonal anti-Troma-III antibody (a gift from Professor R. Kemler, Max Planck Institute of Immunobiology, Freiburg, Germany) for 60 minutes at 37°C in a humidity chamber, followed by incubation with allophycocyanin-conjugated secondary antibodies (donkey anti-rat IgG) diluted to 1:200 for the next 60 minutes. 0.05 % PBS-Tween buffer pH 7.6 was used for

the washing steps as needed.

Immunhistochemical staining for alpha-smooth muscle actin (ASMA)

To perform the (ASMA) staining, 5 μm paraffin sections were mounted on slides, dried, dewaxed and antigen-retrieved in a pressure cooker for 1-5 minutes at 120°C. Then the sections were incubated with the mouse anti-ASMA primary antibody MSK030 diluted 1:300, detected with ZytoChem-Plus alkaline phosphatase (AP) Polymer-Kit and the enzyme was visualized using FastRed Kit (both from Zytomed Systems GmbH, Berlin, Germany). The staining Kits were used according to the manufacturer's instructions. The sections were counterstained with hematoxylin.

Control samples

Tin order to prepare the control samples, sections of tissues perfused without CFDA-SE were processed, or PBS was used instead of the primary antibody.

Preparation of the single cell suspension from the liver

For the preparation of a cell suspension, the liver was perfused with 5-7 ml of 180 μm CFDA-SE-Solution in DMEM and then immediately placed in 10 ml of enzyme solution containing 360,000 U/ml collagenase (Sigma C0130), 1 g/l bovine serum albumin (Sigma A8022), 1.5 mg/l αD (+)-Glucose (Sigma G-6152), 2 mM L-Glutamin (Biochrom K 0282), 100,000 U/l Penicillin G (Biochrom A2213), 3% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 700,000 U/l Hyaluronidase type III (Sigma H2126 type II), 36 mM 4-(2-hydroxyethyl)-1-piper-azine-ethanesulphonic acid (Biochrom L1613) und 130,000 U/l DNAse (Deoxyribonuclease I, E.C. 3.1.21.1) (Roche Diagnostics GmbH, Mannheim, Germany) in L-15 Medium (Leibovitz) (Sigma L1518) for 15 minutes, to remove the connective tissue capsule. The solution was decanted and new enzyme solution was added.

Then the organ was minced into small pieces of 1 mm^3 with a sterile scalpel and gently shaken for 15 minutes at 37°C in a water bath. The cell suspension was pipetted for 1 min to allow the release of liver cells into the medium. The digestions were repeated 3 to 5 times and the cell suspensions were passed through 100, 70, 40 and finally a 30 μm nylon Cell Strainer (BD Biosciences, USA). The cell suspension was centrifuged for 5 min at 600g, 4°C and the cell pellet resuspended in 5 ml DMEM and kept at room temperature in the dark until FACS analysis. The control tissues were perfused with DMEM without addition of CFDA-SE-solution.

Flow cytometry analysis and cell sorting

Cell sorting experiments were performed with a FACSVantage® SE cell sorter (Becton Dickinson (BD) Immunocytometry Systems, 2350 Qume Drive, San Jose, CA95131, USA).

Two parameters were chosen to select the cells: the cells had to be strongly fluorescent for CFDA-SE in the green channel (optical filter: bandpass 530nm center wavelength ±15nm) and their forward scatter signal had to be greater than the forward scatter signal from 6 μm beads (Accudrop beads, BD Biosciences, 2350 Qume Drive, San Jose, CA95131, USA). For excitation, a 488nm laser (Model: Enterprise 621 from Coherent Inc. 5100 Patrick Henry Drive Santa Clara, CA 95054 USA) was used. To determine the autofluorescence level of CFDA-SE untreated cells in the green channel, liver cells were measured using cell suspensions from a DMEM perfused mouse livers. The sorted cells were collected into 15 ml tubes containing 5 ml DMEM supplemented with 10% FBS. Cell suspensions were centrifuged for 5 min at 600 g, 18°C and resuspended in 2 ml DMEM. One part of each suspension was assessed by trypan blue exclusion to control the viability of cells. The remaining cells were cytospun onto slides and immunostained for CK-19.

Results

Vascular system after supravital perfusion with CFDA-SE

The greater part of non-adherent blood cells was eliminated by perfusion of the blood vessels with a warm heparin solution to sustain continuous inflow of the CFDA-SE solution to all parenchymal organs and muscles.

Under *in vitro* conditions, CFDA-SE needs approximately 8 minutes to cross the cellular membrane (Wang et al., 2005). Therefore, we perfused all animals on a heating plate to prolong vitality and metabolic activity of the stained cells.

By testing the influence of exposure times and different concentrations of CFDA-SE solution on the efficiency of blood vessel perfusion, well detectible fluorescence was obtained by application of 30 ml of 100 μM solution of CFDA-SE for 10 minutes. Observation of tissue sections showed green fluorescence inside the blood vessels and small capillaries within liver, kidney, heart, brain and femoral muscles. The endothelium was intensely fluorescent while the smallest capillaries were rendered visible by single cells. Fluorescence of the intima was stronger in arteries than in veins. The fluorescence of smooth muscle cells was not detectable, but very weak extravascular fluorescence was observed when the organ pressure was overcome during perfusion or perfusion time was prolonged at greater than 20 minutes (Fig. 1).

Biliary tree after supravital perfusion with CFDA-SE

Successful perfusion of the biliary tree in mice was achieved by infusion of CFDA-SE solution retrogradely through the gall bladder. Cropping of the liver margins allowed retrograde outflow of the perfusate through opened bile canaliculi and small bile ducts and thus

facilitated the injection of large volumes of labeling solution without strongly affecting liver architecture. In initial experiments parts of the liver parenchyma were not always successfully perfused. This difficulty was solved by venting of the perfusion system to completely eliminate air bubbles preventing the dye solution from being equally distributed throughout the liver parenchyma. After perfusion the fluorescent CFDA-tissue conjugates were detected in the biliary tree as well as in bile canaliculi. The main problem using CFDA-SE was to find the optimal concentration and exposure time to achieve a high fluorescence with low background.

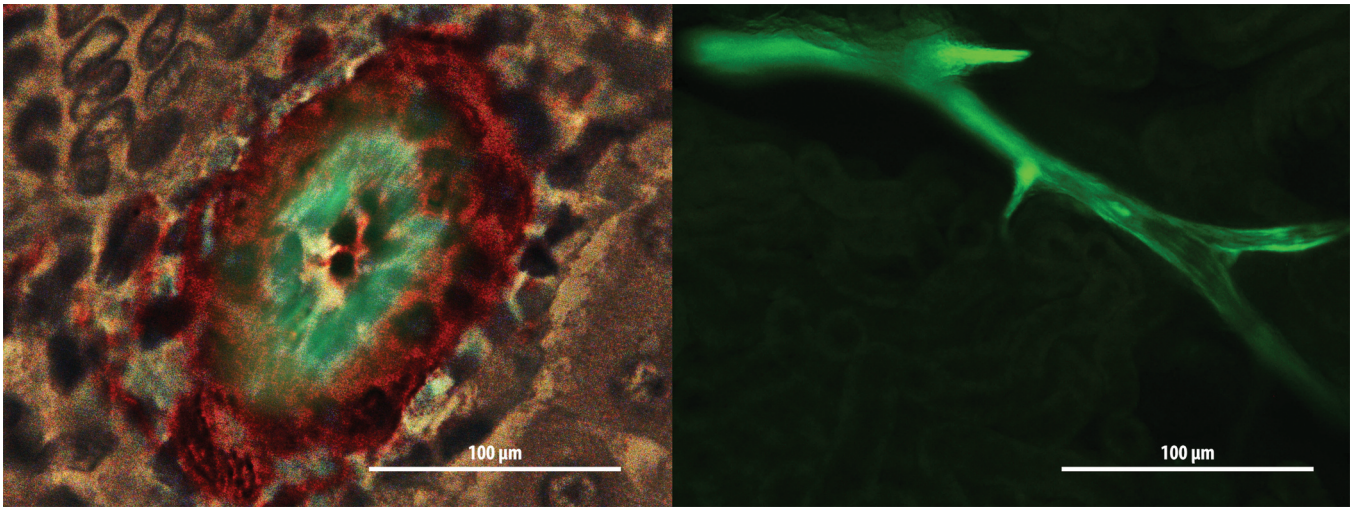

Fig. 1. Merged image of transmitted-light (for ASMA-staining, red) 5 μm thick paraffin section of rat liver **(A)** and fluorescence image of 50 μm thick vibratome section of rat kidney **(B)**, showing green fluorescence in blood vessels after perfusion with CFDA-SE solution through the abdominal aorta.

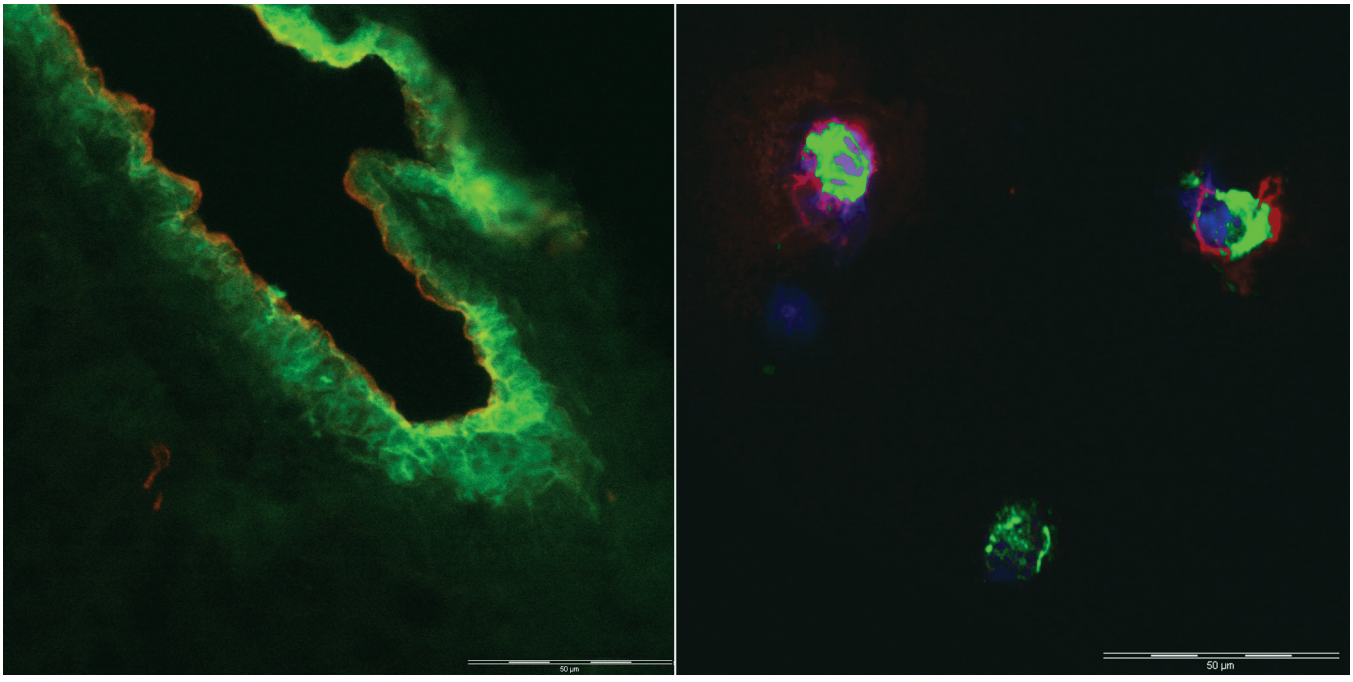

Fig. 2. Merged fluorescence image of a bile duct in a 10 μm thick mouse liver cryosection **(A)** and cytospin of cells harvested by FACS-sorting **(B)**, obtained from mice livers after retrograde perfusion with CFDA-SE through the gall bladder, showing the fluorescence of CFDA-conjugates (green) and CK-19 immunostaining (red). The cytospinned cells were counterstained with DAPI (blue).

After testing different concentrations, it was found that a 180 μM solution of CFDA-SE produced the best result for the visualization of cholangiocytes. The strongest fluorescence was found in the large- and medium-sized bile ducts. Small bile ducts were less intensely fluorescent.

A comparison of fluorescence patterns showed that, to a large extent, the pattern of fluorescence of CFSE-conjugates and that of CK-19 immunohistochemistry originated from the same structures. In some areas of liver tissues, very slight fluorescence of hepatocytes was apparent, but this was generally not significant (Fig. 2, left).

Separation of CFDA- SE labeled cells from liver cell suspension

The CK-19 positive cells were identified as primary cholangiocytes. Their fraction in the cytospins prepared from the liver cell suspension was less than 1%. Microscopic examination of cytospins prepared from FACS separated cell fractions revealed that the concentration of primary cholangiocytes was 5% by separation using fluorescence and cell size parameters exclusively. This proportion rose to 20-40% if cells were sorted using cell size parameters and fluorescence intensity simultaneously (Fig. 2, right). Immediately after separation, cell viability was determined and amounted to more than 80%.

Discussion

The aim of this study was to develop a novel, reliable and straightforward alternative technique for labeling of the biliary tree and liver vasculature, as well for the histological demonstration and isolation of bile duct epithelial cells. This was achieved by using the cell tracer CFDA-SE for retrograde perfusion of bile ducts or blood vessels of mice and rats, respectively. These small rodents were chosen as the most frequently used experimental animal models of hepatic fibrosis (Constandinou et al., 2005; Iredale, 2007; Hayashi and Sakai, 2011).

In our study, we showed, for the first time to our knowledge, the usefulness of CFDA-SE for supravital staining of biliary and vascular tree in situ. For that purpose, a new technique of retrograde perfusion of the biliary tree was established: the injection of a liquid through the gall bladder and the cropping of the liver margins allowed free out-flow of the perfusate and thus prevented the deformation and damage of the biliary tree. The perfused tissue samples were subjected to immunohistochemical staining: the fluorescence of CFDA-SE continued to be strongly detectable after acetone-, formalin- or alcohol fixation, in paraffin- and cryosections. In contrast to this stable labeling with CFDA-SE the identification of cells by specific antibodies as cell-specific markers is affected severely by various methods of tissue processing.

Upon contact with the cell membrane, CFDA-SE quickly penetrates into the cytoplasm, where the amine-reactive carboxyfluorescein succinimidyl ester triggers a strong fluorescent signal that is covalently bound and thus prevents the substance from being washed out by histological procedures. As dead cells lack intracellular esterase activity, CFDA-SE labels live cells only (Riordan et al., 1994; Wang et al., 2005; Boyd et al., 2008). In our material, the CFDA-SE tissue-conjugates stained cells lining the luminal surface of blood vasculature and bile ducts, representing a practical tool for the observation of lining cells, as well as permitting an estimated differentiation between live and apoptotical cells in all vascular compartments.

The perfusion of CFDA-SE marked tissues with Microfil® did not interfere with fluorescence, thus allowing the use of the same tissues for magnetic resonance imaging as well as for histological examination.

This technique also represents a useful tool for tissue engineering or cancer research, where three-dimensional (3-D) imaging of bile ducts or vessel sprouts in addition to the detection of specific cellular markers is a very common requirement.

In practice, some techniques such as corrosion casting have already been described and are used to visualize biliary and blood vessel anatomy (Aharinejad and Lametschwandtner, 1992). The scanning electron microscopy of corrosion casts provides strong, precise information about the three-dimensional architecture networks and intimal cell patterns of the investigated tissue, but tissue perfused with casting materials, for example with Mercox®, could thus far not be used at the same time for other histological procedures.

Marking of vessels or bile ducts with CFDA-SE represents an alternative method for their quick visualization at a substantially lower price than by using specific antibodies. The complete perfusion takes no longer than 30 min. A further advantage of the capability of CFDA-SE to stain cells without affecting their vitality is the opportunity for the concomitant use of the same tissues for both morphological and *in vitro* studies.

In this study, vital cholangiocytes were separated from the normal mouse liver cell suspension using fluorescence-activated cell sorting. The current methods of isolation of vital cholangiocytes from the mouse liver are afflicted with a plethora of technical difficulties in obtaining sufficient numbers of viable cholangiocytes, especially in mice: cholangiocytes represent just 2-5% of the resident liver cells (Cho et al., 2001; Alpini et al., 2003; Chen et al., 2004; Pietersen et al., 2004). No cell surface markers specific for mouse cholangiocytes are described or are commercially available: both EpCam and CD133 are expressed in oval cells (Cardinale et al., 2011; Rountree et al., 2011). The amount of CK-19 positive cells in the collected cell fractions was up to 20% with respect to all cells in the sorted fraction. The isolated CK-19 positive cells were different in size. Based on the studies of Alpini at al., we speculate that

both small and large cholangiocytes can be separated by this method (Alpini et al., 1998; Glaser et al., 2009, 2010).

The CFDA-SE is not a specific marker for cholangiocytes and it is able to diffuse through the cell junctions of the epithelial layer and co-stain cells surrounding the smallest intrahepatic bile ducts. We speculate that hepatocytes constitute the largest fraction of contaminating cells, and further purification of the cholangiocyte fraction should be in the focus of future studies.

In summary, for the first time, a fast, simple and cost-saving method of retrograde perfusion and intravital fluorescent labeling of bile duct epithelium and blood vessels using CFDA-SE has been developed and validated. This procedure opens up new possibilities for morphological research, monitoring of liver damage, and isolation of vital cholangiocytes. We believe that it can increase successful verification of experimental results, as well as decrease the use of animals in laboratory experiments.

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