In situ assessment of the liver microcirculation in mechanically ventilated rats using Sidestream dark-field (SDF) imaging.

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Short title

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Summary

Assessment of hepatic microcirculation by on-line visualization has been impossible for a long time. Sidestream dark-field (SDF) imaging is a relatively new method allowing direct visualization of both mucosal microcirculation and surface layers microcirculation of solid organs using hand-held probe for direct contact with target tissue. The aim of this study was to evaluate the feasibility of studying the rat hepatic microcirculation in situ by SDF

imaging. The liver lobes were left in situ, and images were obtained using SDF imaging on the surface of the liver via upper midline laparotomy. Images were captured intermittently during 10-sec apnoea and recorded. The microvascular parameters were compared with previous validation studies. Clear high contrast SDF images were successfully obtained. Quantitative analysis revealed a mean FSD (functional sinusoidal density) of 402±15 cm/cm^2, a sinusoidal diameter of 10,2±0,5 μm and postsinusoidal venular diameter of 33,9±13 μm. SDF imaging is a suitable noninvasive method for accurate quantification of the basic microcirculatory parameters of the liver in situ without a need to exteriorize the liver lobes. This method seems to be applicable in animal studies with possibility to use SDF imaging also intraoperatively, providing unique opportunity to study liver microcirculation during various experimental and clinical settings.

Key words

SDF imaging – liver perfusion – intermitent positive pressure ventilation – rats

Introduction

A wide variety of insults affecting the liver (e.g. sepsis, ischemia-reperfusion injury or hypovolemia) can induce the changes in the microvascular regulation, resulting in regions with compromised perfusion leading to the various clinical consequences (Clemens *et al.* 2005). Although the regulatory mechanisms of hepatic sinusoidal blood flow has not been fully understood, previous animal studies have demonstrated that effective restoration of microvascular perfusion is an essential determinant of the liver function recovery (Chun *et al.* 1994). For many years, intravital microscopy (IVM) of the liver was the only one powerful experimental method to investigate hepatic microcirculation under physiological and pathological conditions allowing direct visualization of the microcirculatory network of the

liver. Orthogonal polarization spectral (OPS) imaging and its successor sidestream dark-field (SDF) imaging are relatively new, progressively developing, noninvasive optical methods with potential to directly visualize microcirculation based on very similar principles, which have been described in details previously (Groner *et al.* 1999, Schiessler *et al.* 2002, De Backer 2003, Ince 2005). Briefly, in OPS imaging green (550±70 nm) polarized light, guided through a system of lenses, illuminates the target tissue to achieve optimal imaging of microcirculation because at this wavelength oxy- and deoxy-hemoglobin absorb the light equally. The light reflected at the surface is eliminated by orthogonal polarizer (analyzer) and does not form the image. The light after undergoing multiple scattering becomes depolarized and passes through the polarizer to create an image of the underlying microcirculation. SDF imaging is based on slightly different principles as compared with OPS technology. Light-emitting diodes (LEDs) arranged in a ring formation at the tip of the light guide emit green light (540±50 nm) which directly illuminates the tissue microcirculation. The illuminating light source is optically isolated from the emission light path in the core of the light guide (Figure 1). Hence, SDF technology provides improved resolution and clarity of the images compared to OPS imaging. Both SDF and OPS technology have been implemented in a hand-held videomicroscope convenient for both experimental and clinical conditions. Validation studies which have been reviewed recently have shown a statistically significant agreement of the data obtained from new optical technologies and IVM (Cerny *et al.* 2006). The SDF imaging method could represent a unique tool for assessment of hepatic microcirculation both in experimental and human studies.

The purpose of the experimental study was designed to elucidate whether SDF imaging in anesthetized mechanically ventilated rats can be used for direct imaging of hepatic sinusoidal perfusion and quantitative assessment of the basic physiological hepatic

microcirculatory parameters in vivo. The results were compared with current available validation studies and possible further clinical applications are discussed as well.

Methods

Animals. All experimental procedures were performed after Ethical Board approval in accordance with Czech legislation on the protection of animals. Nine male Wistar rats (Bio-Test, Konarovice, Czech Republic), weighing 310 – 350 g, were included in the study. They were housed in groups of two in a standard cage at 21°C in a 12 hr dark/12 hr light cycle and were supplied with unrestricted amounts of laboratory chow (Velaz, Prague, Czech Republic) and tap water. After one week acclimatization period, the rats were enrolled into the study.

Anaesthesia and surgical preparation. After overnight fasting with unrestricted access to tap water, the rats were anesthetized with an intraperitoneal induction dose of pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL, USA; 50mg/kg of body weight.). The animals were placed in a supine position on an operating table, rectal temperature was kept at 36,5 – 37,5°C by use of a heating lamp. The carotid artery and femoral vein were cannulated with polyethylene catheter 24G for continuous blood pressure monitoring, continuous infusion of normal saline (20ml/kg/hour) and to administer additional anesthesia and muscle relaxant pipecuronium bromide (Arduan, Richter Ltd, Budapest, Hungary; 0,2mg/kg of body weight) as necessary. The animals were tracheotomized (Vasocan 14G, B. Braun Melsungen AG, Germany) and mechanically ventilated (ventilator Harvard Scientific, Boston, USA, ventilatory setting: tidal volume = 10 ml/kg, PEEP = 2 cm H_2O, respiratory rate = 60-70/min, FiO_2 = 0,4). Mean arterial blood pressure (MAP) and rectal temperature were recorded every 5 minutes in each animal throughout the study.

Different surgical model from those used widely in animal studies previously (Kondo *et al.* 1998, Langer *et al.* 2001, Marzi *et al.* 1990, Menger *et al.* 1991, Terajima *et al.* 1999,

Vollmar *et al*. 1994) was used for assessment of hepatic microcirculatory changes using SDF technology in rat. After having shaved their abdomen an upper midline laparotomy was performed to visualize left and right liver lobes. Liver lobes were kept *in situ* without any mechanical manipulation throughout the study. Then the wound was covered by sterile 37°C warmed normal saline-saturated cotton swabs to minimize dehydration and lost of body heat. A waiting period of 20 min followed to confirm the stability of arterial blood pressure and rectal temperature (Langer *et al.* 2000). SDF imaging then was performed according to protocol.

Sidestream dark-field imaging procedure. To minimize artificial pressure and movement artifacts, which is crucial (Lindert *et al.* 2002), the SDF imaging probe (MicroScan Video Microscope, Microvision Medical, Inc., Amsterdam, The Netherlands) was attached to a custom made (Arrow International Czech Republic, a.s.) flexible arm with special adapter allowing micromovement of the SDF probe in various axes according to inclination of the flexible arm (Figure 2) and proper stabilization of the probe at the same time. In an effort to objectify the SDF imaging of microcirculation as much as possible, the following methodology was established: once a sector for imaging was selected, the SDF imaging probe was placed approximately 0,5 mm above the target tissue using a flexible arm, then the arm was fixed and the probe covered with plastic lens was moved towards tissue by the adapter for micromovement. Immediately after the first contact with the investigated organ – liver lobe, the focus ring of the probe was used to bring the proper layer into focus to create sharp and high contrast image appropriate for later off-line analysis. The sites of interests on the surface of the left and right liver lobe were selected radomly. In order to eliminate movement artifacts and lateral movement of the liver caused by mechanical ventilation at once and to keep the liver lobes in situ, all SDF images were recorded during 10 seconds lasting apnea (turning off ventilator). Each captured sequence was followed by 2 minutes of stabilizing period. Any

exposed tissues, apart from those covered by SDF imaging probe at the given moment, were intermittently moisturized using warmed sterile normal saline at 37°C. SDF images were obtained from three different areas within the site of interest as recommended recently (Boerma *et al.* 2005). All SDF imaging data of the microcirculation were digitally recorded. At the end of the experiment the animal was killed by an overdose of pentobarbital.

Off-line analysis. Off-line selection of the most stable clips with clear images for final analysis was performed. To summarize, a total of 18 selected clips (6 clips of each area) of 10 seconds were analyzed per each animal. The final on screen magnification of the images obtained with the SDF imaging device was 325 times original. Basic microcirculatory parameters such as functional sinusoidal density (FSD), sinusoidal diameter and postsinusoidal venular diameter were obtained using AVA V1.0 software (AMC, University of Amsterdam, The Netherlands).

The following parameters were analyzed off-line:

1. Functional sinusoidal density (FSD) given in cm/cm^2
2. The diameter of liver sinusoids of the midzonal segment of a liver acinus given in *mm*
3. The diameter of postsinusoidal venules given in *mm*
4. Small (<15*m*m in diameter) vessel rate **x** 100 given in ***%***

Statistics. The descriptive data are presented as mean (95% confidence interval of mean; SD - standard deviation). Statistical analyses were performed with the use of SIGMASTAT 2.0 (Jandel Scientific, San Rafael, CA, USA).

Results

A total of 9 rats were used in this study. Main cardiovascular parameters were stable throughout the study with MAP of 100±6 mm Hg. No circulatory instability was observed during on-line SDF imaging in short-term apnea. With special custom made fixation device

for the SDF probe, clear high contrast images were successfully obtained from the surface of the rat liver lobes in situ using SDF imaging technology. The morphological structures of hepatic microvaculature were clearly identified and it was possible to perform basic off-line quantitative measurements of microcirculatory parameters in mechanically ventilated rat. Typical images with characteristic structure of a liver acinus surface captured using SDF imaging are shown (Figure 3). Results are given in mean (95% confidence interval of mean; Standard Deviation). The mean FSD obtained from the surface of the liver was 402 (399 – 406; 14,4) cm/cm^2, mean sinusoidal diameter was 10,2 (10,1 – 10,3; 0,36) μm and postsinusoidal venular diameter 34,0 (30,0 – 38,0; 13,2) μm. The percentage analysis of small vessels (small vessel rate) has shown a value of 96,2 (94,0 – 98,4; 7) %.

Discussion

This study has confirmed the reliability and accuracy of the SDF imaging when assessing hepatic microcirculation. The values of basic microcirculatory parameters are compatible with those published in validation studies previously (Langer *et al*. 2000). SDF imaging optical technology is a relatively new noninvasive method for direct on-line visualization of microcirculation without a need to use phototoxic fluorescent dyes (Saetzler *et al.* 1997), which allows the application of this method both in experimental and clinical studies.

On-line visualization and recording of hepatic surface microcirculation was performed during short-term apneic pause (8-10 seconds) to avoid exteriorization of the liver lobe minimizing tissue injury and to simulate potentially real clinical conditions when using SDF imaging intraoperatively. The effect of this short apneic pause on hepatic microcirculation in rat cannot be excluded but no hemodynamic instability during monitoring MAP and heart

rate was observed and the results were not different from those published previously (Langer *et al*. 2000, Langer *et al.* 2001).

Regulation of liver microcirculation with regard to dual circulation is very complex. The hepatic artery and the portal vein circulation join to perfuse the hepatic sinusoids. Although the sinusoids, like capillaries in other vascular beds, do not contain vacular smooth muscle cells, it is commonly accepted the fact, that they are subject to active constriction (Zhang *et al.* 1995, Zhang *et al.* 1994) mediated by specialized contractile pericyte called the hepatic stellate cell (HSC) (Bauer *et al.* 2000). Under physiological conditions, the HSC contracts in response to endogenous endothelins, but not α-adrenergic agonists such as phenylephrine and relax in response to nitric oxide (NO) and carbon monooxide (Zhang *et al.* 1995). Endothelin expression is upregulated during endotoxemia, hypoxia, or ischemia. (Sonin *et al.* 1999). However, the mechanisms by which endotoxemia or ischemia induce hepatic microcirculatory dysfunction are not fully understood. Thus accurate sinusoidal diameter measurement is of special importance both for future experimental and clinical studies. Our study demonstrates high accuracy in sinusoidal diameter visualization and measurement when using SDF imaging compared to previous validation studies mentioned above. The value of FSD reflects nutritional tissue perfusion and it is indirect parameter of oxygen delivery to the tissue (Harris *et al.* 1996). In our opinion, determination of FSD represents the preferable method for the SDF imaging technique, because determination of sinusoidal perfusion rate is impossible due to the basic principle of the method that does not allow the visualization of non-red blood cells – perfused sinusoids.

Another modality affecting splanchnic microcirculation is artificial pulmonary ventilation with intermittent positive pressure ventilation (IPPV) which is accompanied by decreased cardiac output (CO) (Saner *et al.* 2006, Marini *et al.* 1980) and alteration of microcirculation in connection with decreased CO was also described (De Backer *et al.*

2004). Though IPPV is a component of the protocols of a number of experimental and clinical studies using the techniques of OPS or SDF imaging, the possible effect of IPPV is not mentioned in the interpretation of the results. The value of positive end-expiratory pressure (PEEP) is another measured variable that must be taken into account when assessing and interpreting changes at the microcirculatory level. As published previously, fluid resuscitation can reverse the undesirable effect of PEEP on CO, but only partial correction of the negative effect of PEEP on mesenteric blood flow can be achieved (Love *et al.* 1995). Stable value of PEEP 2 cm of H_2O was maintained throughout presented study thus PEEP is not supposed to be a fundamental variable affecting significantly hepatic microcirculation in this study.

Despite further technological improvements several limitations of this method remain (Lindert *et al.* 2002). Artificial pressure of the SDF probe and lateral movement of the tissue are the most important factors affecting accuracy of the measurement of microcirculatory parameters. Special custom made device allowing micromovement of the probe, standardized procedure of the visualization with image focusing and short apneic pause were used in this study to minimized artifacts mentioned above, however the possibility to avoid any micromovements of the probe at clinical settings is limited. Majority of microcirculatory parameters has to be analyzed off-line due to software limitation, further software upgrade will be desired in the course of putting the method into clinical practice.

The technology of SDF imaging has been incorporated into a small hand-held improved video-microscope, which can be used in various clinical setting. Human studies using noninvasive optical technologies OPS or SDF imaging have been mainly conducted on nailfold (Mathura *et al.* 2001), brain (Pennings *et al.* 2006, Mathura and Ince 2000), cutis of term and preterm infants (Genzel-Boroviczeny *et al.* 2002), sublingual mucosa in critically ill patients (Sakr *et al.* 2004) and liver during liver resection and transplant surger (Puhl *et al.* 2003, Puhl *et al.* 2005). Due to high quality of the SDF images comparable with those

obtained via IVM, their accurate quantitative analysis and easy manipulation, SDF technology should be considered as very useful and promising research tool for understanding of pathophysiology of liver microcirculation both in experimental and clinical setting (e.g. intraoperative studies assesing hepatic nutritional blood flow during abdominal surgery). Using SDF imaging of the liver in various clinical scenarios could elucidate the regulation and behaviour of hepatic microcirculation (e.g. effect of intraoperative fluid therapy, effect of different anesthetic management or various types of hepatic and non-hepatic surgical techniques) also with regard to clinical course and patient‘s outcome. SDF imaging is a reliable powerful noninvasive method with potential to affect developing new therapeutic strategies that target sinusoidal diamaters or FSD based on future experimental and clinical studies assessing physiology and pathophysiology of the liver microcirculation.

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Figure 1 – Sidestream dark-field (SDF) imaging, an optical scheme

(1) Green light is emitted by (2) peripheral 540 ± 50 nm light-emitting diodes (LEDs) toward tissue arranged in a circle at the end of the light guide. The microcirculation is directly illuminated from the side by green light absorbed by hemoglobin of erythrocytes which are observed as (3) dark moving cells. The imaging, central part, of the light guide (4) is optically isolated from the LEDs. A Magnifying lens (5) projects the image onto a camera (6).

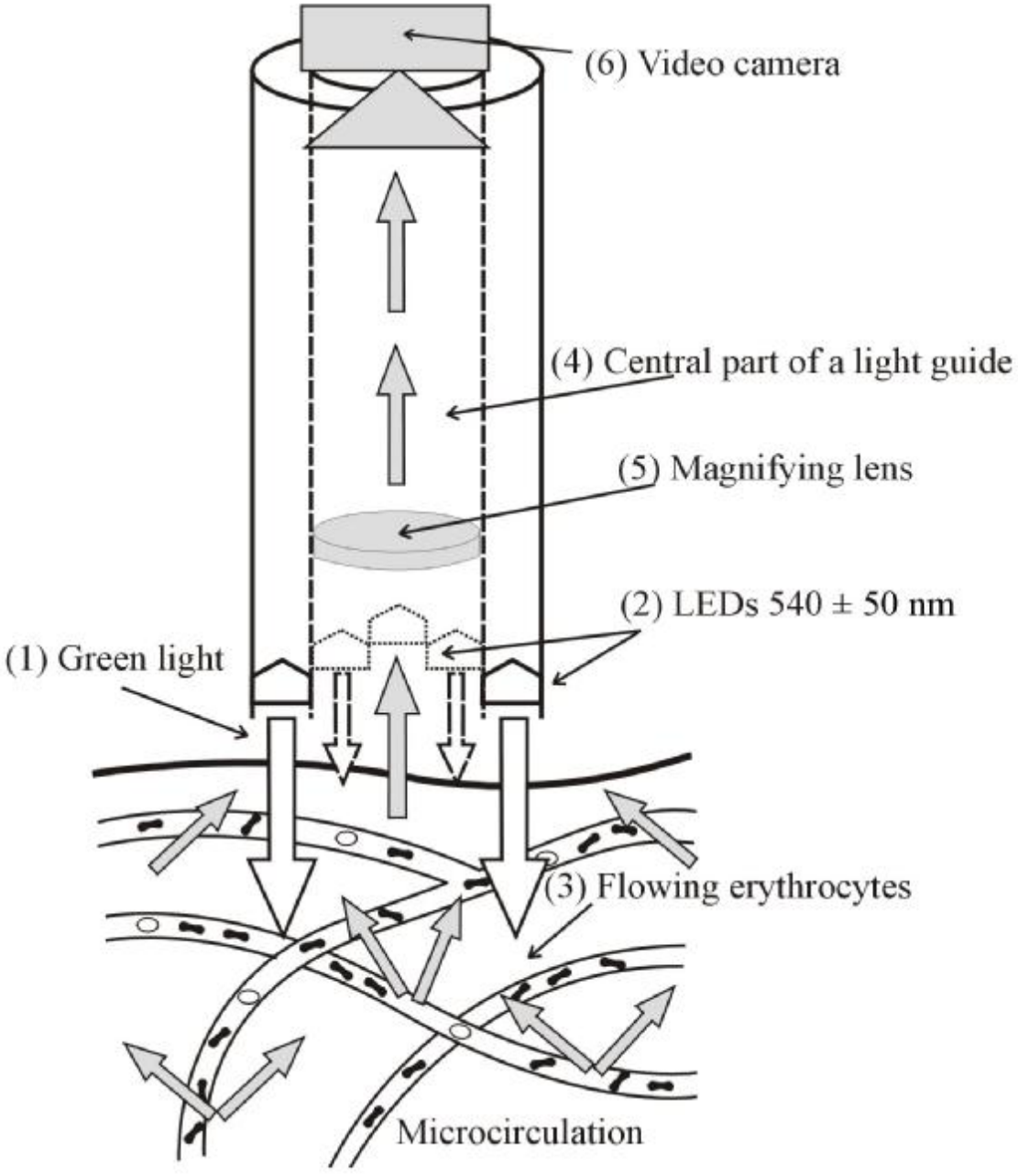

(6) Video camera
(4) Central part of a light guide
(5) Magnifying lens
(2) LEDs 540 ± 50 nm
(1) Green light
(3) Flowing erythrocytes
Microcirculation

Figure 2 – A scheme of the stabilizing and fixation device for SDF imaging probe
(1) Flexible arm allowing horizontal, vertical and rotating movements ends with a special adapter with (2) micrometer screw for movement of the SDF probe (3) with MicroScan lens (4) within the range of 0,5 mm towards the target tissue (5).

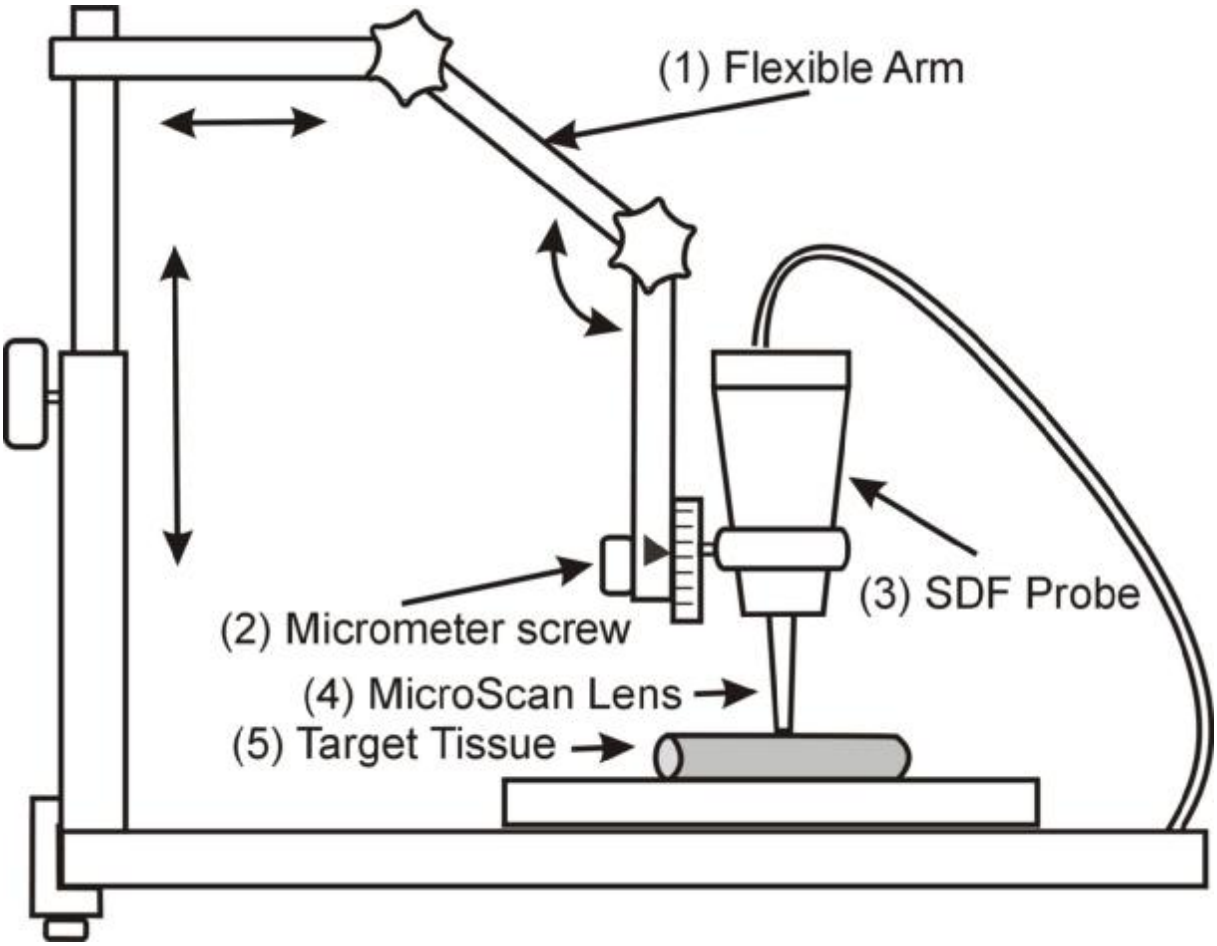
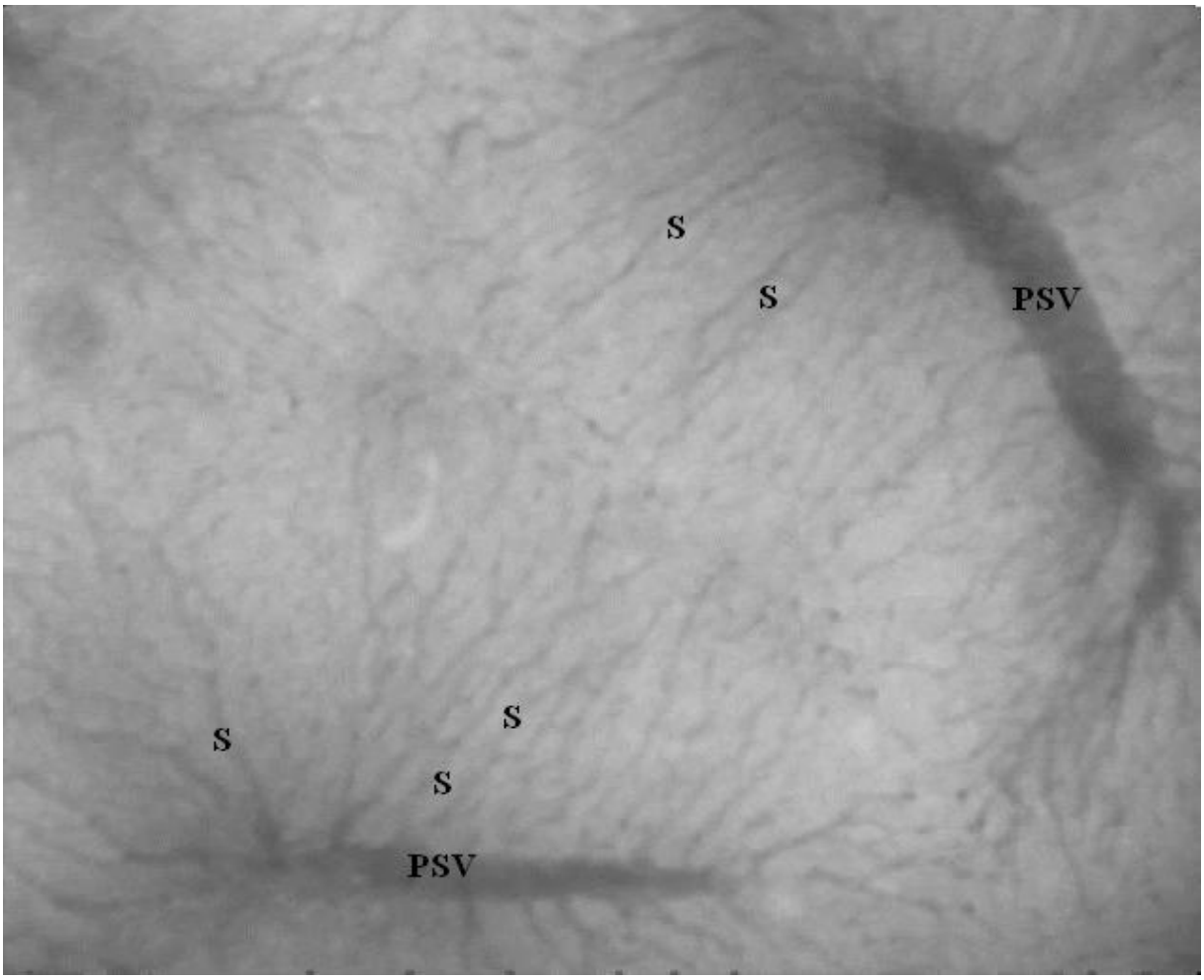


Figure 3 – SDF representative image of the rat liver microcirculation



Sidestream dark-field (SDF) imaging of the the rat hepatic microcirculation.

Objective 5×, on screen 325×

S - liver sinusoids, **PSV –** postsinusoidal venules