Video Article

Intravital Microscopy Imaging of the Liver following *Leishmania* Infection: An Assessment of Hepatic Hemodynamics

Sreekanth Dasari[1], Pascal Weber[1], Camélia Makhloufi[1], Erica Lopez[2], Claire-Lise Forestier[1]

[1]INSERM 1095, URMITE, University of Aix-Marseille

[2]U.F.R de Médecine, University of Aix-Marseille

Correspondence to: Claire-Lise Forestier at claire-lise.forestier@inserm.fr

URL: https://www.jove.com/video/52303
DOI: doi:10.3791/52303

Keywords: Immunology, Issue 101, Intravital microscopy imaging, mouse liver, blood flow labeling, blood flow quantification, *Leishmania* infection, mCherry

Date Published: 7/28/2015

Citation: Dasari, S., Weber, P., Makhloufi, C., Lopez, E., Forestier, C.L. Intravital Microscopy Imaging of the Liver following *Leishmania* Infection: An Assessment of Hepatic Hemodynamics. *J. Vis. Exp.* (101), e52303, doi:10.3791/52303 (2015).

Abstract

Intravital microscopy (IVM) is a powerful optical imaging technique that has made possible the visualization, monitoring and quantification of various biological events in real time and in live animals. This technology has greatly advanced our understanding of physiological processes and pathogen-mediated phenomena in specific organs.

In this study, IVM is applied to the mouse liver and protocols are designed to image *in vivo* the circulatory system of the liver and measure red blood cell (RBC) velocity in individual hepatic vessels. To visualize the different vessel subtypes that characterize the hepatic organ and perform blood flow speed measurements, C57Bl/6 mice are intravenously injected with a fluorescent plasma reagent that labels the liver-associated vasculature. IVM enables *in vivo,* real time, measurement of RBC velocity in a specific vessel of interest. Establishing this methodology will make it possible to investigate liver hemodynamics under physiological and pathological conditions. Ultimately, this imaging-based methodology will be important for studying the influence of *L. donovani* infection on hepatic hemodynamics.

This method can be applied to other infectious models and mouse organs and might be further extended to pre-clinical testing of a drug's effect on inflammation by quantifying its effect on blood flow.

Video Link

The video component of this article can be found at https://www.jove.com/video/52303/

Introduction

Organ-specific hemodynamics are important physiological features of any mammalian organ. Abnormalities in blood flow may be the consequence of inflammation and a sign of organ dysfunction[1]. Thus, blood flow organization, structure and function appear as critical parameters for analysis under physiological and pathological conditions. The techniques that have been commonly used for analyzing blood flow in a specific organ contain several limitations, including the resolution limit of the technique itself (*e.g.* Doppler imaging of blood flow), the capacity for measurement of absolute blood flow only (volume of blood per unit serving an organ) (*e.g.* Optical Coherence tomography) and the measurement of average changes in speed in a large and heterogeneous population of blood vessels[2,3]. The liver's circulatory system links different vessel subtypes that are heterogeneous in their size, structure and function. In this study, intravital microscopy (IVM) imaging technology is applied to evaluate liver hemodynamics *in vivo,* in real time, at high resolution and in parallel to uncover the characteristics of the individual blood vessels that comprise the hepatic organ. The recent development of this powerful optical imaging technique allows the researcher to collect dynamic data on living animals at a high spatial and temporal resolution. By allowing the direct visualization and real time monitoring of specific and rapid biological processes *in vivo*, IVM provides a unique opportunity to the researcher to image individual blood vessels, and measure and quantify the velocity of single red blood cells (RBC) within a specifically selected hepatic vessel.

In this study, we have implemented the IVM technique in the mouse liver to investigate the influence of mouse infection by the hepatotropic *Leishmania* parasite on liver hemodynamics. *L. donovani* is the agent responsible for visceral leishmaniasis, a severe disease characterized by acute-on-chronic inflammatory responses and a pathology that is present in multiple organs, including the spleen and the liver. In an experimental mouse model of visceral leishmaniasis, the liver infection is self-resolving whereas the splenic infection is progressive[4]**.** These outcomes of *Leishmania* infection with respect to the individual organs are still not completely understood. Investigation of liver and spleen hemodynamics under pathological conditions will shed new light on host-parasite interactions and disease pathogenesis.

Our experimental model system is based on exposing and imaging the liver of an anesthetized mouse that received intravenous injection of specific fluorescent dyes for the labeling of the hepatic intravasculature. The liver is a favorable organ for intra-vital microscopy. After performing a small incision in the abdomen, the liver is gently externalized and placed on wet gauze, then on a coverslip with the goal of reducing any motion artifacts due to heartbeat and respiration. The liver is then placed within the view of a microscope lens. As compared to the spleen and

lymph node which require the use of two photon microscopy for IVM studies, the advantage of the liver lies in its homogenous 3D architecture/anatomy that allows for the use of a conventional confocal microscope, with a maximum penetration depth of approximately 50 µm, for intravital microscopy imaging[5-8].

This study describes two independent imaging methods for the quantitative measurement of RBC velocity and blood flow speed in individual blood vessels. In the first method, liver blood flow is acquired using a xy bi-dimensional mode over time. The resulting xyt data are analyzed using the MtrackJ plugin in the free ImageJ software, which allows for the tracking of individual RBCs over time. In the second method, a single blood vessel is selected and its corresponding blood flow is analyzed using the line scanning fast acquisition mode of the confocal laser-scanning microscope. The vessel of interest is scanned at high frequency along its central axis through an axial line. The blood flow velocity is then quantified based on the difference in contrast between unlabeled dark erythrocytes and fluorescently labeled plasma. The fluorescence intensities of RBCs and plasma acquired along the line scan are plotted against time to obtain streaks, the angles of which are proportional to the velocities of an individual RBC.

The goal of this article is to provide a simple and reproducible method for imaging and measuring blood flow velocity within individual blood vessels of the liver and to make available the basic tools for the successful performance of mouse surgery, IVM and quantitative analyses of the velocity of individual RBCs. This approach will allow researchers to gain new insights into blood velocity under pathological conditions.

Protocol

Ethics statement: All the animal studies were performed in accordance with guidelines and protocols that were approved by the Institutional Animal Care and Use Committee of the Aix-Marseille Université, France. Female C57Bl/6 mice at 8 - 10 weeks old were commercially obtained and handled according to the rules of Décret N° 8 87–848, October 19, 1987, Paris. All the experiments using *L. donovani* LD1S parasites were conducted in accordance with biosafety regulations from the French and European Union legislation.

1. Mouse Infection with *L. donovani* Promastigote Parasites

1. Prepare the culture medium for *in vitro* maintenance of *L. donovani* promastigote parasites, LD1S (MHOM/SD/62/1S-CL2D).
    1. Use M199 medium. Supplement M199 medium with 10% fetal calf serum (heat-inactivated at 56 °C for 30 min), 25 mM HEPES pH 6.9, 12 mM $NaHCO_3$, 1 mM glutamine, RPMI 16040 vitamin mix, 10 mM folic acid, 100 mM adenosine, 7.6 mM hemin, 50 U/ml penicillin and 50 mg/ml streptomycin.
2. Culture LD1S parasites in 10 ml of complete M199 medium at 26 °C in a 25 cm^2 ventilated flask.
3. Using successive differential centrifugation steps, prepare a population of promastigote parasites that are enriched in metacyclic forms.
    1. Harvest a mid-log phase parasite culture. Count on a hemocytomer. Resuspend 5 x 10^5 parasites/ml in 10 ml of complete M199 medium. Grow the parasite culture in anaerobic conditions until they reach the late stationary phase (in approximately 5-6 days).
    2. Spin the LD1S culture at 1,200 x g for 10 min at 26 °C to pellet non-metacyclic parasites, recover the supernatant and spin it again at 2,500 x g at 26 °C for 10 min.
    Note: This final pellet is highly enriched in metacyclic promastigote parasites.
    3. Resuspend the pellet in 100 µl of PBS. Remove a small aliquot for fixation with 25% glutaraldehyde 1/100 v:v and count parasites using a hemocytometer.
4. Dilute the enriched-metacyclic parasite population to 1 x 10^7/100 µl in PBS. Inject the parasite suspension into the tail vein of an anesthetized mouse (see below) using a 1 ml syringe with a 30 G needle. For control animals, inject 100 µl PBS into the tail vein.

2. Surgical Procedures

1. Disinfect the working space with a biocidal disinfectant spray and all the surgical instruments with 70% ethanol for 30 min.
2. Prepare an anesthetic solution, according to the weight of the animal, containing 125 mg/kg of Ketamine and 12.5 mg/kg of Xylazine diluted in PBS.
3. Weigh the mouse and intraperitoneally inject the appropriate dose of anesthetic using a 1 ml syringe with a 30 G needle.
4. Keep the mouse warm and check that the mouse is completely anesthetized by pinching the foot pad before proceeding. Re-inject a half dose of the anesthetic solution in the mouse every 60 min.
5. Shave the mouse's abdomen and clean it with Vetedine. Make a small incision in the skin and musculature under the thoracic cage on the left side of the abdomen.
6. Lay a piece of moist gauze on the abdomen just below the opened area. Expose the liver and place it on the gauze. Lay another piece of moist gauze above the liver.
7. Place a 24 x 60 mm^2 to 150 µm thick coverslip, cyanoacrylate-bonded to a metal frame, on the liver for visualization under a microscope.
8. Protect the eyes of the mouse with a wet gauze.
9. After the imaging session, euthanize the anesthetized animal by cervical dislocation.

3. Intravital Microscopy Imaging of the Liver Architecture

1. Carry out the intravital microscopy experiments on an inverted confocal microscope equipped with a thermostatic controlled chamber, an apochromatic 63X oil glycerol immersion objective (NA 1.4), Argon (488 nm) and HeNe (543 nm, 633 nm) lasers and a blue diode (405 nm).
2. Place the mouse on the stage of the microscope with the coverslip covering the liver face down on the objective. Set the temperature of the chamber to 29 °C. Adjust the focus using the liver autofluorescence to allow for visualization of the sinusoids.

3. To visualize the vasculature, prepare a solution of 500 µg BSA-Alexa 647 diluted in 100 µl of PBS, for each mouse. Inject this solution intravenously into the tail vein of the anesthetized mouse using a 1 ml syringe with a 30 G needle.
4. To visualize the liver cell nuclei, prepare a solution of Hoechst 33342 in PBS. Inject this solution at 8 mg/kg of mouse intraperitoneally using a 1 ml syringe with a 30 G needle.
5. Use the normal sequential mode, the 63X immersion objective and the scanner at 400 Hz to image the hepatic cell nuclei and the liver vasculature. Turn on the 405 nm blue diode for Hoechst excitation and the 633 nm laser for BSA-Alexa 647 excitation. Turn on the Argon 488 nm laser and define a large acquisition band to visualize the liver autofluorescence.

4. Intravital Microscopy Imaging of the Liver for Blood Flow Speed Measurement

1. Open the LAS software of the microscope (LAS-AF viewer Version 3.1.0 build 8587). Select the resonance scanner mode when opening the microscope software.
2. Click on the configuration tab to set the hardware. Click on laser and select the HeNe 633 laser. Click on settings and select 12 bits resolution.
3. Click on the acquisition menu. In the beam pathway settings window, select the objective 63X and set up the 633 laser power to 100%. Activate the signal gain and off set parameters by selecting Alexa-633 from the PM1-3 drop down menu.
4. In the acquire tab, select the 'xyt' acquisition mode. Set format width at 1,024 x 1,024. Select speed at 8,000 Hz. Select the pinhole of 3 Airy units. Select a zoom factor of 3. Do not select the bidirectional mode.

5. Quantitative Analysis of RBC Velocity Using the xyt Images (Method 1)

1. Click on 'Live' icon to visualize the blood flow on a live mode. Choose the area of interest and select a specific blood vessel for analysis. Set the vessel of interest to horizontal position by adjusting the 'X', 'Y' and scan field rotation co-ordinates on USB control panel.
2. Stop the 'Live' mode once the vessel of interest is set. Change the format set width to 1,024 x 256 in the acquisition mode setting window, line average = 1, frame average = 1, select “stacks” 150. Click on 'start' to acquire images.
3. For quantitative analysis of RBC velocity using the “xyt” images open the ImageJ software. Select the 'File' tab in ImageJ, click “Open” then choose the file of interest.
4. Go to the “plugin” menu in ImageJ, select LOCI and Bio-formats import options. In this window, select the stack viewing parameters as 'hyperstack'. In the stack order option, select the option: “xyzct”. Note: This opens a window with all the series of acquisitions.
5. Click on 'Plugins' on the ImageJ menu and select MTrackJ option. Click on 'add' icon on the small window and click on the RBC to track. Click on the same RBC in each following frame and click on 'measure' icon to measure the speed.
   Note: The file generated can be saved in .exl format.
6. Track a minimum of five RBCs per vessel and analyze a minimum of three individual vessels of the same size.

6. Quantitative Analysis of RBC Velocity Using the xt Line Images (Method 2)

1. Click on 'Live' to visualize the blood flow in live mode. Select a specific blood vessel for analysis. Set the vessel of interest to horizontal position. Stop the 'Live' mode once the vessel of interest is set.
2. Select the 'xt' scanning mode and set the central lumen of the selected vessel along the line scan. Set format width at 1,024 x 512, speed = 8,000Hz, line average = 32, time = 512. Click on 'start' and acquire the xt line images.
3. Generate streaks for quantitative analysis of RBC velocity by opening the .lif file and opening the xt line image of interest. In LAS-AF software, select “experiments”, open the .lif and select the image. Note: It will automatically open a kymograph.
4. Measure the time (t, obtained on the Y axis) required for a particle streak (showing dark reflection) to travel a certain distance (d, obtained on the X axis) on this kymograph. Select the tool “draw line” and draw a line horizontally to the streak. Note the distance in µm and the time in seconds generated in the result tab at the bottom of the image.
5. Calculate velocity as V = distance/time using the values obtained from the kymograph.
6. Quantify a minimum of five particles streaks per xt image and a minimum of three individual vessels of the same size.

Representative Results

The specific architectural organization of the sinusoids in the liver can be visualized based on the autofluorescent property of this organ (**Figure 1**, panel B and C, green), the intraperitoneal injection of Hoechst for the labeling of hepatocyte nuclei (**Figure 1B**, blue) and the intravenous injection of fluorescent BSA for the staining of the hepatic circulatory system (**Figure 1C**, red). The liver is composed of several different vessel subtypes with different functions and structures and sizes ranging from 40-700 μm^2 in diameter (D) (D of small vessels is between 40-80 μm^2 and D of large vessels is above 300 μm^2). Intravital microscopy imaging of the liver allows for the visualization at high resolution of the heterogeneity and complexity of the liver vasculature (**Figure 1C**).

To monitor blood flow speed in individual vessels, two different methodologies are used for the image acquisition and data analysis of the liver blood vessels *in vivo*. In the first method, real-time imaging of the blood flow in a vessel of interest is performed using the xyt scanning mode (**Movie 1**). Quantitative analysis of the velocity of an individual RBC in a single vessel is performed using the MTrackJ plugin in the ImageJ software (**Figure 2A** and **2B**). A minimum of five particles per vessel is tracked in different vessels ranging from 40-80 μm^2 of diameter. The results obtained with this method give consistent and reproducible values across different mice and evaluate the RBC velocity in small liver vessels at values between 25-35 µm/sec (**Figure 2C**).

The second method consists of repeatedly scanning along a line parallel to the vessel wall, with the line placed in the center of the vessel lumen. **Figure 3** shows a xy frame scan (left panels) with the corresponding xt line scan (right panels). The velocity of an individual RBC is given by the ratio V = distance/time and is calculated using the orthogonal projections on the X and Y axis of the streak obtained in the xt image. In **Figures**

3D and **3E**, each data point represents the velocity of an individual RBC from different small or large vessels selected from different mice. As seen from the error bars, the data show a relatively low variation between RBC velocities and indicate that blood flow speed is dramatically faster in large vessels than in small vessels. In addition, a significant variation in the velocity values obtained from different types of large vessels was observed whereas blood flow speed is relatively similar across different types of small vessels. Blood flow measurements with this newer method generate data that is comparable to data obtained from the previously described method using MTrackJ (compare **Figure 2C** to **Figure 3D**). The image in **Figure 3C** represents a typical example of a suboptimal experiment in which the xy frame scan generates an un-interpretable xt image. In this particular case, the problem results from both i) the fast internalization of BSA into the endothelial cells lining the vessel of interest and ii) the presence of a junction between two vessels that perturbs the blood flow in the selected area of acquisition. The rate of internalization of the plasma reagent into the endothelium lining the blood vessel is a critical parameter to consider when measuring blood flow speed, as internalization will alter the quality of the xt image that shows the streaks. The internalization rate critically depends on the dose of the dye that is intravenously injected. In **Figure 4** we show that 500 µg of BSA-Alexa 647 and/or 500 kDa of Dextran-FITC are the optimal doses that should be used for the quantification of blood flow speed. These doses generate a very bright signal that allows for the identification of the RBC as dark particles against a bright background and enable the visualization of blood flow for a minimum of 1 hr without internalization of the injected dye (**Figure 4**, bottom panel). In contrast, 50 µg of BSA-Alexa 647 and/or 500 kDa Dextran-FITC (not shown), while allowing for the visualization of the liver's vascular network, is a suboptimal dose for measuring blood flow velocity due to the very fast internalization of the dye, which starts at 5 min post-injection (**Figure 4**, bottom panel).

When applying this methodology to the liver of infected animals, we observed a significant increase in the blood flow velocity as soon as 1 hr post-infection. Alteration of the RBC velocity is maintained up to 24 hr post-infection. It ultimately reaches normal values at 72 hr post-parasite injection (**Figure 5**). Thus, this experiment validates the use of this methodology to gain new insights into the influence of pathological conditions on liver hemodynamics.

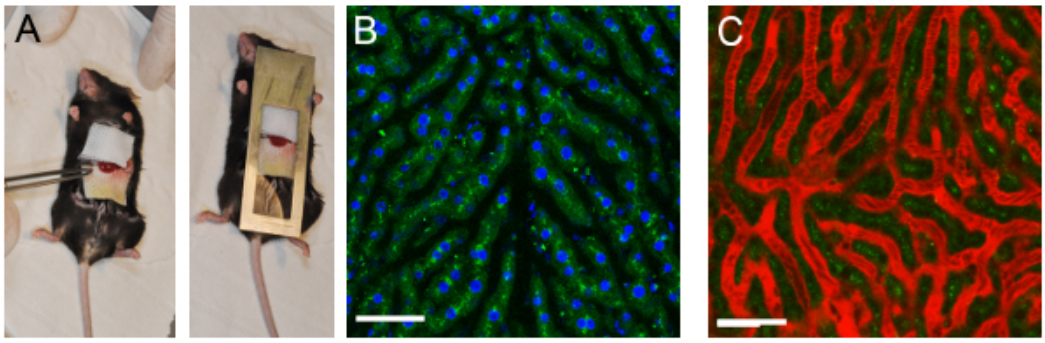

Figure 1: Intravital Microscopy of the Mouse Liver. (**A**) Liver surgery of the mouse. A small area of a liver lobe is exposed after surgery, two moist gauze pieces are placed around it and a slide is put on the abdomen to cover the liver prior to the mouse being placed on the stage of a confocal microscope. (**B**, **C**) Image of a representative area of the liver of a non-infected mouse that demonstrates the liver sinusoids and the different sizes of the vessels that comprise the hepatic organ. Liver autofluorescence is shown in green. The mouse is injected with Hoechst 33342 (blue) to label the hepatocyte nuclei (**B**) and injected with BSA-Alexa 647 (red) to visualize the liver vasculature (**C**). Scale bars, 50 µm Please click here to view a larger version of this figure.

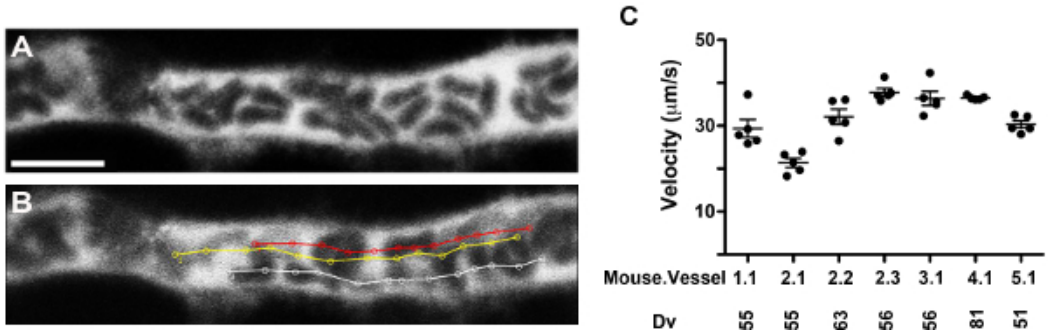

Figure 2: Quantitative Analysis of RBC Velocity from a xy Frame Scan using the ImageJ Software. (**A**) Representative xy image of blood circulation in a small liver vessel acquired by intravital microscopy imaging of the mouse liver using the fast xy scanning mode. Dark areas correspond to RBCs. Scale bar, 10 µm. (**B**) Quantification of the velocity of a single RBC using the MTrackJ plugin from ImageJ. Lines (red, yellow, white) represent the trajectory over time of an individual RBC. (**C**) RBC velocity in individual small (D is approximately 50 μm^2) hepatic vessels. The graph plots the value of the velocity of five independent RBCs tracked in individual liver vessels that were selected from five different mice (1 to 5). D represents the diameter of the vessel in μm^2. Please click here to view a larger version of this figure.

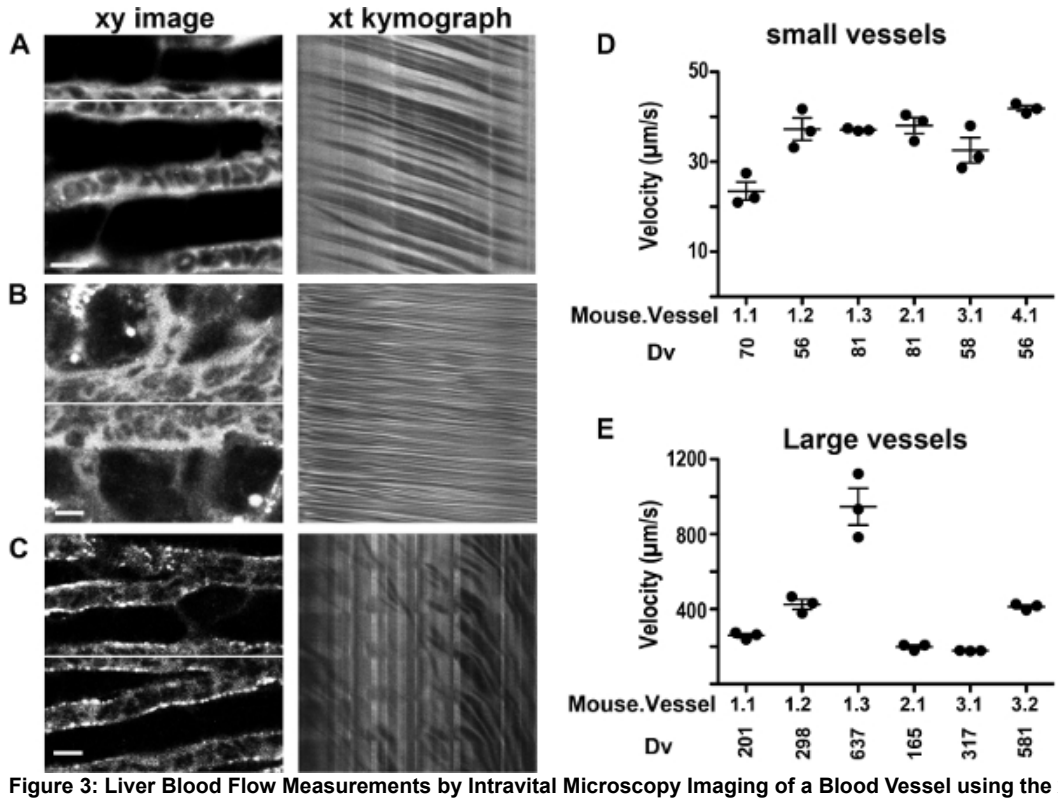

Figure 3: Liver Blood Flow Measurements by Intravital Microscopy Imaging of a Blood Vessel using the xt Line Scanning Mode. Representative xy images (left panels) of liver vessels labeled with BSA-Alexa 647 and their corresponding xt images (right panels) obtained from a line-scan of the central lumen of an individual vessel. (**A**, **B**) Small (**A**) and large liver vessel (**B**). (**C**) Representative data from a sub-optimal experiment showing the intersection of two blood vessels and BSA internalization into the endothelium. (**D**, **E**) The graphs plot the value of the velocity of three independent RBCs per vessel (small in D and large in E) selected from five different mice. D represents the diameter of the vessel in μm^2. Please click here to view a larger version of this figure.

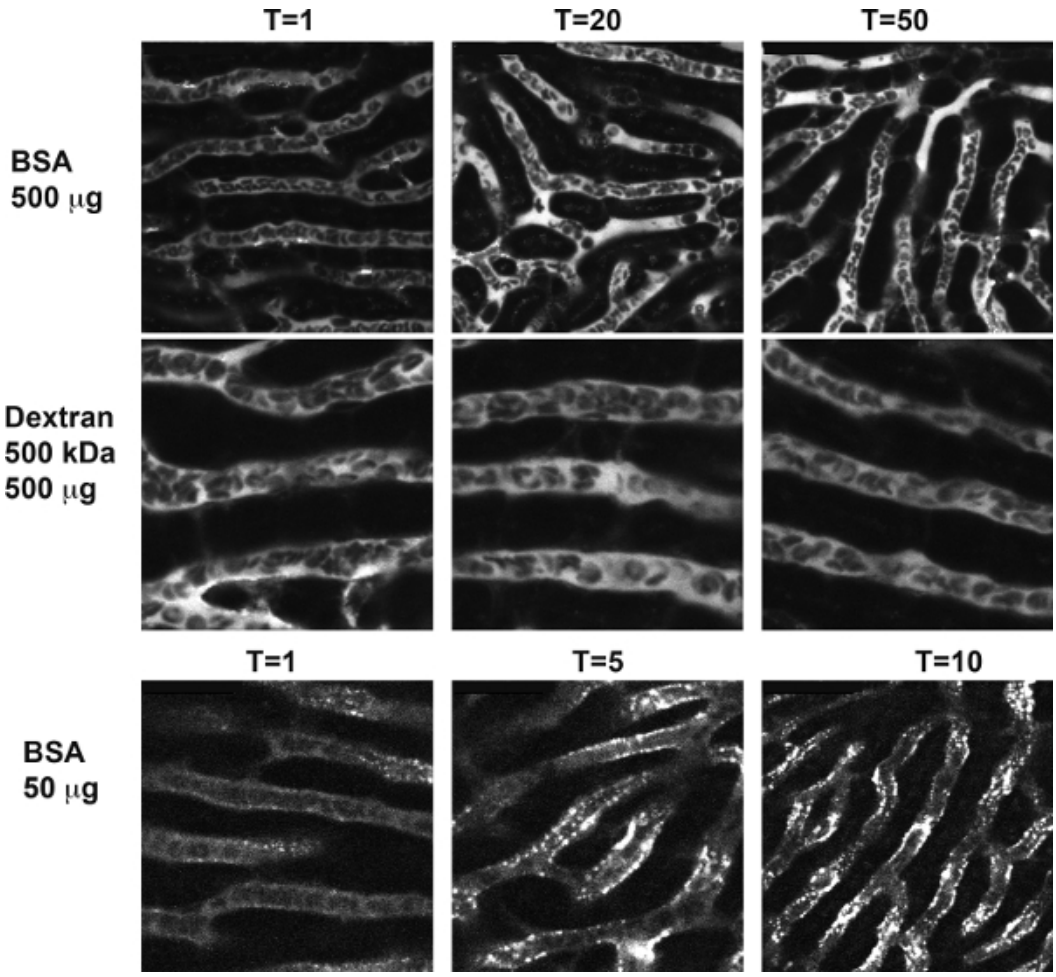

Figure 4: The Effect of the Dose of the Fluorescent Plasma Dye on Its Internalization into the Endothelium Lining the Sinusoids. This image tracks the internalization of BSA and Dextran 500 kDa into the endothelium lining the sinusoids, as a function of time and injection dose. Please click here to view a larger version of this figure.

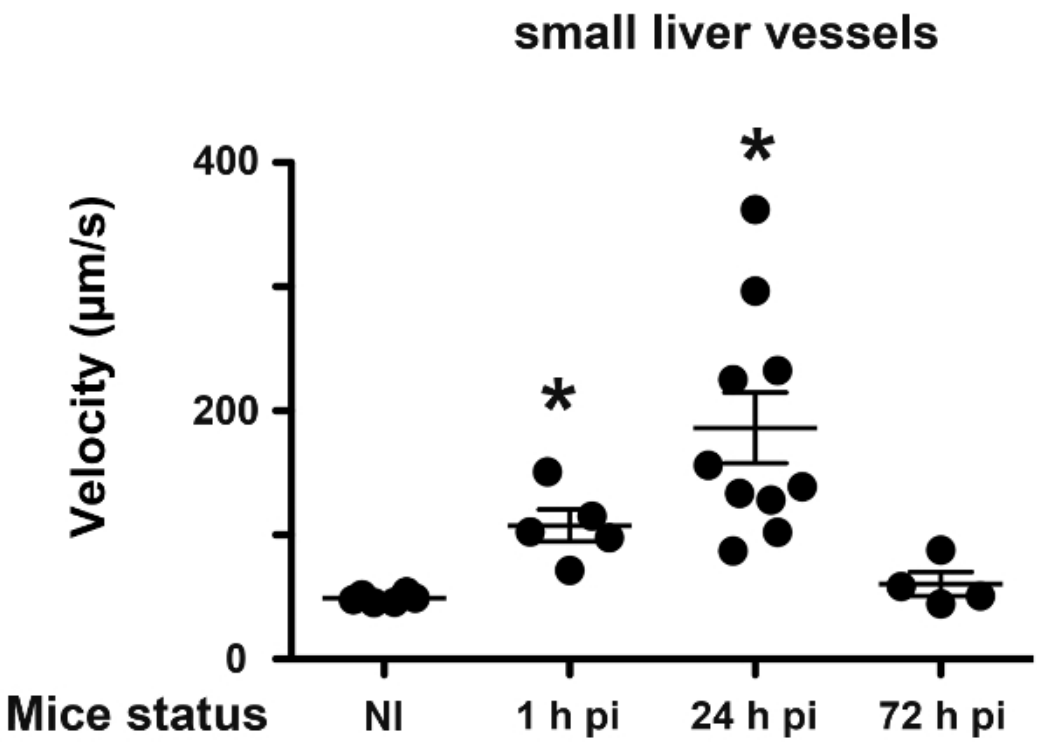

Figure 5: Effect of *L. donovani* infection on Liver Hemodynamics. This graph plots the values of the blood velocity obtained from several individual blood vessels from non-infected mice (NI) and mice that were infected with *L. donovani* and analyzed 1 hr, 24 hr and 72 hr post-infection (pi). Each dot represents the mean value of five RBC velocity measurements obtained in a single blood vessel of approximately 50 μm^2. In each condition (NI, 1 hr pi, 24 hr pi and 72 hr pi) a minimum of three mice were analyzed for blood flow speed. The stars indicate a significant difference in the blood flow speed obtained 1 hr and 24 hr pi compared to NI mice ($P < 0.01$, one way Anova).

Movie 1.Time-lapse Intravital Microscopy Imaging of the Mouse Liver. **Movie 1** corresponds to **Figure 3**. The mouse received an injection of BSA-Alexa 647 to visualize the vasculature. Blood flow in three small vessels (D of 50 μm^2) is shown. Black areas correspond to RBC.

Discussion

The recent development of intravital microscopy of the mouse liver opens up new possibilities for the investigation of physiological response to infection *in vivo* and in real time[5,9,10]. Organ blood flow is a critical physiological parameter that is often altered in many diseases. However, the status of liver hemodynamics under physiological and infectious conditions remains a poorly explored area. In this study, IVM-based methods, which were previously adapted for the study of the spleen and tumor vasculature, were implemented to quantify liver blood flow speed within a single vessel and measure individual RBC velocity *in vivo*[11,12]. The methodologies used here rely on the contrast between a plasma fluorophore injected intravenously in the mouse, such as fluorescent BSA, and the erythrocytes, which remain dark. *In vivo* blood flow measurements have the potential to improve our understanding of disease processes.

Here, two methodologies were used and compared to measure blood flow in liver vessels. The first methodology is time consuming and fastidious in term of data analysis as it requires users to manually track RBC movement in an individual vessel using the MTrackJ plugin in the ImageJ software. The second methodology is simple and fast, as it consists of the analysis of an xt image of a blood vessel generated from a line scan acquisition with resonant scanner mode. The two methodologies give comparable data for similar vessels. At this stage it is almost impossible to compare our data with others, as, to our knowledge, blood flow speed has never been quantified in such small vessels and in mouse sinusoid capillaries with a width of approximately 10 µm. IVM-based methods allow high resolution in xy of 500 nm enabling the analysis of a sinusoid with a width of 10-50 µm [13]. In contrast, Doppler-based techniques only have a maximal resolution of 70 µm, thus limiting the measurement of the blood flow to liver arteries and portal veins [3].

One of the main steps that is critical for the successful implementation of these methodologies is a successful surgery and maintenance of the anesthetized mouse in good physiological condition during the entire imaging session. To do so, one must carefully control the temperature of the microscope chamber and inject anesthetic every hour. Another critical step is the internalization of the fluorescent plasma reagent into the endothelium lining that can occur naturally after its injection. Such internalization will produce uninterpretable xt images and un-contrasted xy images. This phenomenon depends on the dose of the injected dye and can be prevented by injecting a high dose of the plasma reagent (**Figure 4**).

Importantly, successful intravital microscopy imaging of the liver blood flow requires the consideration of several critical aspects. These include the complexity of the liver vasculature system, the presence of different subtypes of blood vessels containing small and large vessels with different structures and functions, different blood circulations with fast and slow flux and rapid RBC velocities. The main limitation of the methodology that is based on the analysis of a xt image is the speed of acquisition of the vessel and the lack of Z resolution, as the image is acquired in 3D (xyt). More specifically, the line scan rates of standard systems reach a limit of sensitivity with regard to the acquisition of large

blood vessels (diameter above 180 μm^2) that are characterized by very high RBC velocities, which is the case with arteries. In the large vessel, the RBC trajectory can be out of the imaged plane and any components that are perpendicular to the scanned plane will be excluded from the analysis.

The methodology presented here represents an ideal tool for the *in vivo* and real time quantification of several blood flow parameters under various pathological conditions. The investigation of liver hemodynamics will shed new light on disease processes and parasite pathogenesis in a specific organ. Our efforts to gain insights into the liver microvasculature system applied to an experimental mouse model of *Leishmania* infection opens up the possibility for the correlation of several blood flow parameters with parasite multiplication and disease development in this organ, in real time. The further development of reagents, tools and the methodology for the improved characterization of the liver's vasculature system by IVM during infection is of great importance for the study of the influence of parasite colonization on this physiological parameter and, reciprocally, the influence of blood flow on *Leishmania* pathogenesis. This strategy can be potentially extended to other infectious systems where pathogens target the liver.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This research was supported by the INSERM, the University of Aix-Marseille and a Career Development award from HFSPO obtained by CL Forestier.

References

1. Vollmar, B., Menger, M. D. The hepatic microcirculation: mechanistic contributions and therapeutic targets in liver injury and repair. *Physiol. Rev.* **89**, 1269-1339 (2009).
2. Srinivasan, V. Absolute blood flow measured by optical methods. *SPIE Newsroom.* (2011).
3. Seifalian, A. M., Stansby, G. P., Hobbs, K. E., Hawkes, D. J., Colchester, A. C. Measurement of liver blood flow: a review. *HPB. Surg.* **4**, 171-186 (1991).
4. Engwerda, C. R., Ato, M., Kaye, P. M. Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. *Trends Parasitol.* **20**, 524-530 (2004).
5. Beattie, L., *et al.* Dynamic imaging of experimental Leishmania donovani-induced hepatic granulomas detects Kupffer cell-restricted antigen presentation to antigen-specific CD8 T cells. *PLoS Pathog.* **6**, e1000805 (2010).
6. Lee, W. Y., *et al.* An intravascular immune response to Borrelia burgdorferi involves Kupffer cells and iNKT cells. *Nat. Immunol.* **11**, 295-302 (2010).
7. Geissmann, F., *et al.* Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol.* **3**, e113 (2005).
8. Marques, P. E., *et al.* Imaging liver biology in vivo using conventional confocal microscopy. *Nat. protocols.* **10**, 258-268 (2015).
9. Thiberge, S., *et al.* In vivo imaging of malaria parasites in the murine liver. *Nat. protocols.* **2**, 1811-1818 (2007).
10. Vacchina, P., Morales, M. A. In vitro screening test using Leishmania promastigotes stably expressing mCherry protein. *Antimicrob. Agents Chemother.* **58**, 1825-1828 (2014).
11. Ferrer, M., Martin-Jaular, L., Calvo, M., del Portillo, H. A. Intravital microscopy of the spleen: quantitative analysis of parasite mobility and blood. *J. Vis. Exp.* (2012).
12. Kamoun, W. S., *et al.* Simultaneous measurement of RBC velocity, flux, hematocrit and shear rate in vascular networks. *Nat. Methods.* **7**, 655-660 (2010).
13. MacPhee, P. J., Schmidt, E. E., Groom, A. C. Intermittence of blood flow in liver sinusoids, studied by high-resolution in vivo microscopy. *Am. J. Phys.* **269**, G692-G698 (1995).

