

Video Article

# Visualization and Analysis of Blood Flow and Oxygen Consumption in Hepatic Microcirculation: Application to an Acute Hepatitis Model

Kosuke Tsukada<sup>1</sup>, Makoto Suematsu<sup>2,3</sup>

<sup>1</sup>Department of Applied Physics and Physico-Informatics, Keio University

<sup>2</sup>Department of Biochemistry, School of Medicine, Keio University

<sup>3</sup>Exploratory Research for Advanced Technology (ERATO), Suematsu Gas Biology Project, Japan Science and Technology Agency (JST)

Correspondence to: Kosuke Tsukada at [ktsukada@appi.keio.ac.jp](mailto:ktsukada@appi.keio.ac.jp)

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## Abstract

There is a considerable discrepancy between oxygen supply and demand in the liver because hepatic oxygen consumption is relatively high but about 70% of the hepatic blood supply is poorly oxygenated portal vein blood derived from the gastrointestinal tract and spleen. Oxygen is delivered to hepatocytes by blood flowing from a terminal branch of the portal vein to a central venule via sinusoids, and this makes an oxygen gradient in hepatic lobules. The oxygen gradient is an important physical parameter that involves the expression of enzymes upstream and downstream in hepatic microcirculation, but the lack of techniques for measuring oxygen consumption in the hepatic microcirculation has delayed the elucidation of mechanisms relating to oxygen metabolism in liver. We therefore used FITC-labeled erythrocytes to visualize the hepatic microcirculation and used laser-assisted phosphorimetry to measure the partial pressure of oxygen in the microvessels there. Noncontact and continuous optical measurement can quantify blood flow velocities, vessel diameters, and oxygen gradients related to oxygen consumption in the liver. In an acute hepatitis model we made by administering acetaminophen to mice we observed increased oxygen pressure in both portal and central venules but a decreased oxygen gradient in the sinusoids, indicating that hepatocyte necrosis in the pericentral zone could shift the oxygen pressure up and affect enzyme expression in the periportal zone. In conclusion, our optical methods for measuring hepatic hemodynamics and oxygen consumption can reveal mechanisms related to hepatic disease.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/3996/>

## Protocol

### 1. Labeling Erythrocytes with Fluorescein Isothiocyanate Isomer I (FITC)

All the experimental protocols we used were approved by the Animal Care Committee of Keio University School of Medicine.

1. Anesthetize a donor mice, and after making a celiotomy incision withdraw whole blood from the inferior vena cava with a 1-ml syringe containing small amount of heparin. The mouse was immediately euthanized by sodium pentobarbital injection (100 mg/kg i.p.).
2. Wash the whole blood twice with PBS (pH 7.4) by centrifugation for 5 min at 400 g in a swinging-bucket rotor.
3. Dissolve 10 mg of FITC in 5 ml of 100 mM Na<sub>2</sub>HPO<sub>4</sub> and filter it with a membrane having 0.22-μm pores.
4. In a test tube mix 1 ml of the FITC solution with 0.15 ml of 3 mM glucose, 0.25 ml of 180 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1.5 ml of 100 mM Na<sub>2</sub>HPO<sub>4</sub>. Add 0.2 ml of the RBC pellet, and tap the tube for mixing.
5. Keep the tube for 2 hours at 4 °C and then wash the stained RBCs in PBS twice.
6. Put a small amount of the RBC suspension on a slide glass and see if the RBCs look healthy (i.e., that their shape and size are normal) and that the fluorescence intensity is sufficient.
7. Suspend the 0.1 ml of RBCs in 0.9 ml of PBS at pH 7.4, and keep the suspension at 4 °C until injection.

### 2. Preparation of Oxygen-sensitive Dye

1. Dissolve 500 mg of BSA in 10 ml of PBS at pH 7.4.
2. Add 30 mg of Pd(II)-meso-tetra(4-carboxyphenyl)porphine (Pd-TCPP) to the BSA solution and stir overnight.
3. (Optional) Extract BSA-bound Pd-TCPP by using gravity-flow chromatography or a spin column to separate it from free Pd-TCPP.
4. Centrifuge the solution to remove undissolved Pd-TCPP, and filter the supernatant with a membrane filter having 0.22-μm pores.

5. Store 1-ml aliquots in tubes at -20 °C. Avoid repeated freeze-thaw cycles.

### 3. Animal Preparation

1. For microscopic observation of the microcirculation prepare a plastic plate with a hole 20 mm in diameter, and tape a 30-mm-square square cover glass over the hole.
2. Anesthetize a mouse, remove the fur, and prep the skin. Place a catheter in the tail vein for drug injection by using a 30 G needle connected to a 10-cm polyethylene (PE 10) catheter filled with heparinized PBS.
3. After median incision, extend a main lobule of the liver on the plastic plate, and place the mouse in sternal recumbency.
4. Prepare small slips with kitchen wrap (3 mm x 8 mm) and tile them around the edge of the hepatic lobe to inhibit moving of the liver with respiration and keeping it from drying.
5. Observe the hepatic microcirculation under a microscope (with transmitted light), and confirm that the blood flow has no stasis in the field of view for at least 15 min.
6. Slowly inject 0.2 ml of fluorescently labeled RBCs for blood flow observation or 0.2 ml of Pd-TCPP solution for pO<sub>2</sub> measurement. This amount of FITC-labeled RBCs will account for 1/50 of all the RBCs in circulation in the visualized region.

### 4. Blood Flow Visualization

1. Excite the FITC by irradiating it with mercury lamp light that has passed through a bandpass filter (450–490 nm)<sup>1</sup>.
2. Record the fluorescent image with a CCD camera.

### 5. pO<sub>2</sub> Measurement

Pd-TCPP phosphorescence is relatively weak and should be detected with a high-sensitivity detector. All experiments need to be performed in a dark room.

1. The absorption peaks of Pd-TCPP are at 410 nm and 532 nm, so the second-harmonic wavelength 532 nm is recommended for excitation. This wavelength can be generated by a Nd:YAG pulse laser<sup>2</sup>.
2. Feed the beam from the Nd:YAG laser into the appropriate port on the inverted microscope, and adjust the beam spot to a central position in the focal plane. The spatial resolution depends on the laser spot size, which is changed by passing the beam through a pinhole.
3. To detect the phosphorescence, attach a long-pass filter (>620 nm) and a photomultiplier tube (PMT) to the other port of the microscope. The PMT should be sensitive to red wavelengths, especially around 700 nm.
4. Sample the phosphorescence signal (500 points sampled at 200 kHz) and calculate the time constant of the decay by fitting an exponential function to the decay.
5. For calibration prepare two set of samples of Pd-TCPP solution with pO<sub>2</sub> 150 mmHg and 0 mmHg. Add dithionite sodium 1% in volume to sample to produce absence of oxygen. Keep the pH of the samples at 7.4 and the temperature at 37 °C during calibration.
6. Fix the Stern-Volmer constant  $k_q$  and oxygen-free luminescence decay time  $\tau_0$  in the equation (1)<sup>3</sup>. In our case, with Pd-TCPP solutions at 37 °C and a pH of 7.4,  $k_q$  is 374 mmHg<sup>-1</sup> s<sup>-1</sup> and  $\tau_0$  is 0.74 ms. These measurement parameters depend on the equipment (i.e. laser, detector, other optical devices) and the signal processing method, so calibration is needed in each measurement system<sup>4</sup>.  

$$\tau_0/\tau = 1 + k_q \cdot \tau_0 \cdot pO_2 \quad (1)$$
7. Set the mouse on the plate on the microscope stage, observe the hepatic microcirculation, and adjust the position of the target microvessel to the point of the laser spot.

### 6. Hepatitis Model

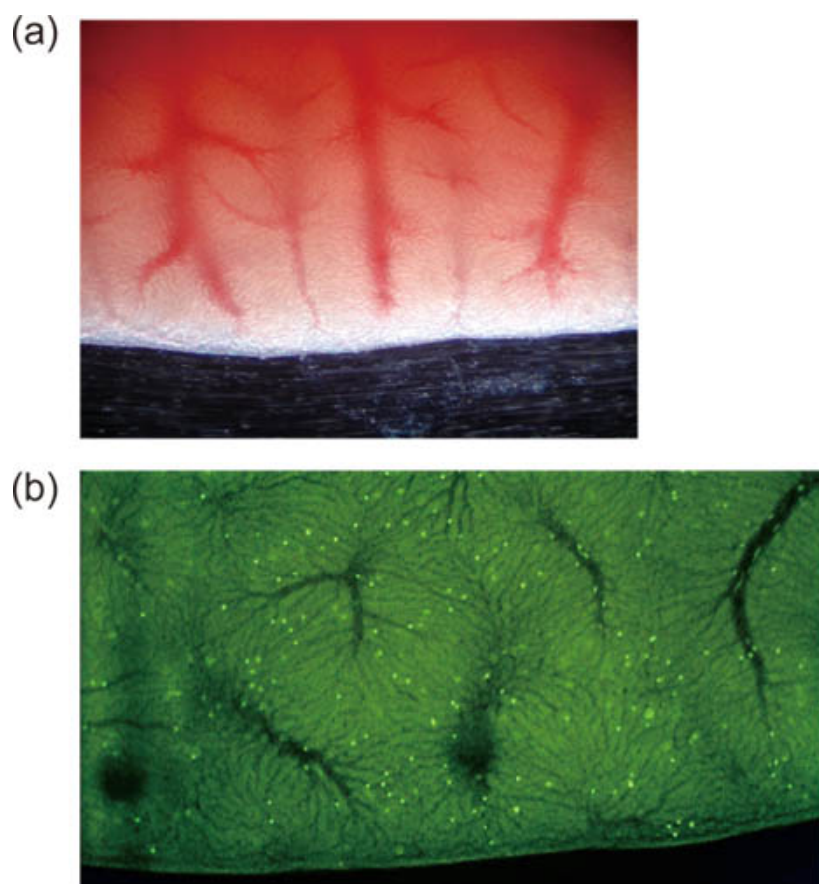
1. Fast mice with free access to water overnight.
2. Prepare a 20 mg per ml solution of acetaminophen (APAP) in DMSO.
3. Inject it intraperitoneally at 1 ml/100 g bw (i.e., 200 mg/kg bw). After the injection, the mouse can be given free access to food and water<sup>5</sup>.
4. 24 hours after injection, anesthetize the mouse and expose the liver by making a median incision. If hepatitis is achieved, necrosis in the pericentral region will be easily visible to the naked eye.

### 7. Representative Results

Example images of hepatic microcirculation are shown in **Figure 1**, where (a) is a transmitted-light image and (b) is a fluorescence image. Individual FITC-labeled erythrocytes are observed in the video movie, and portal venules, sinusoids, and central venules are recognized by blood flow directions.

As shown in **Figure 2**, the laser spot irradiating a central venule through a x100 objective lens was approximately 10 µm in diameter. Intravascular pO<sub>2</sub> in the hepatic microcirculation was measured in male C57BL/6 mice, and **Figure 3(a)** shows the relations between pO<sub>2</sub> and vessel diameter in portal and central venules. The pO<sub>2</sub> gradient from portal to central venules indicates that RBCs release oxygen efficiently while passing through sinusoids. As shown in **Figure 3(b)**, the average pO<sub>2</sub> was 59.8 mmHg in portal vessels, 48.2 mmHg in sinusoids, and 38.9 mmHg in central venules.

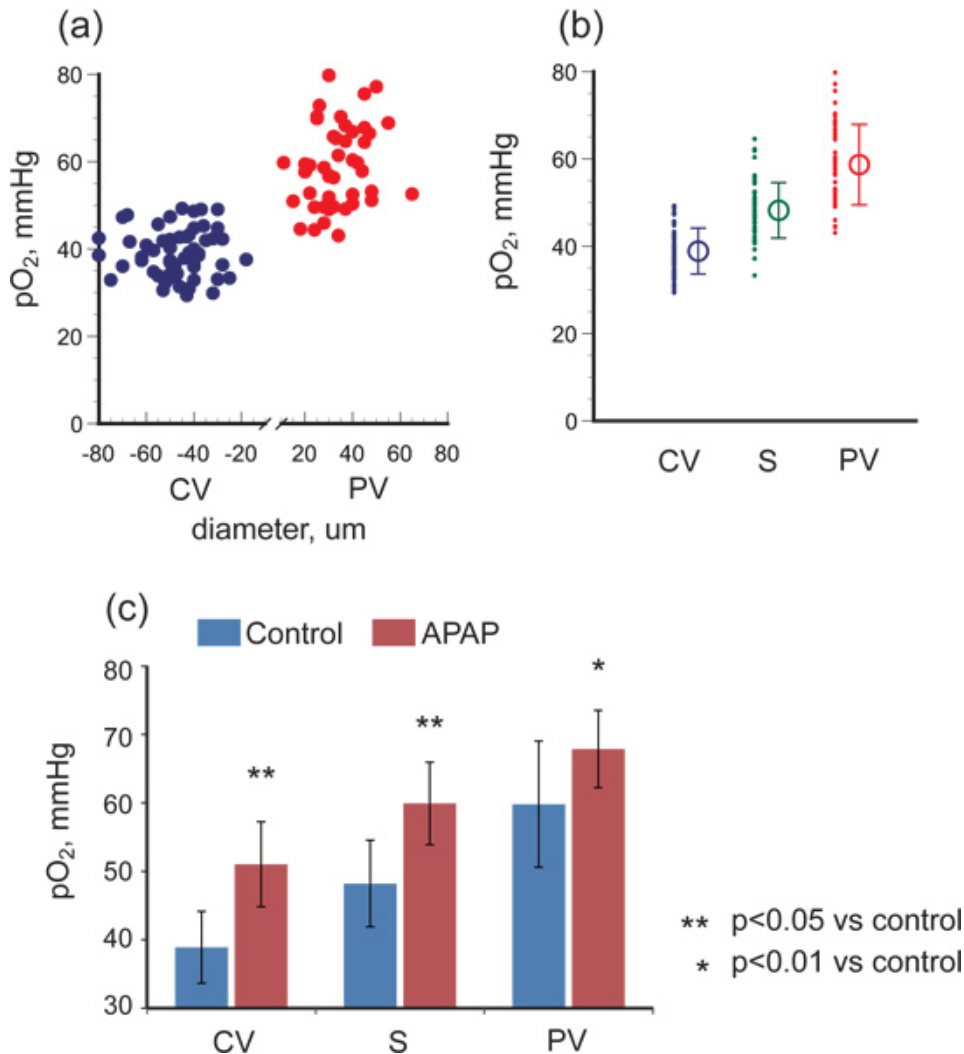
When we produced acute hepatitis by injecting APAP intraperitoneally, intravascular pO<sub>2</sub> was significantly elevated in each part of the hepatic microcirculation: p<0.05 in portal veins and p<0.01 in sinusoids and central veins (**Figure 3(c)**).



**Figure 1.** Low-magnification images showing hepatic microcirculation with (a) transmitted light and (b) fluorescence.



**Figure 2.** Image of laser spot irradiating a targeted central venule for  $pO_2$  measurement.



**Figure 3.** pO<sub>2</sub> gradient in hepatic microcirculation of healthy control mice and of mice with acetaminophen-induced hepatic injury. (a) shows pO<sub>2</sub> versus vessel diameter for portal venules (PV) and central venules (CV), and (b) shows the oxygen gradient from PV to CV via sinusoids (S). The pO<sub>2</sub> difference between PV and CV reflects the oxygen consumption of hepatocytes or other parenchymal cells. (c) shows that in hepatitis conditions induced by APAP administration, pO<sub>2</sub> was significantly elevated in PV, S, and CV and the pO<sub>2</sub> gradient decreased, indicating that hepatic oxygen consumption was compromised.

## Discussion

Delivering oxygen to tissues is an essential role of the microcirculation, and a lot of papers describe diseases related to microvessel anatomy and rheology<sup>6</sup>. Liver blood flow is a combination of arterial and venous blood. About 30% of the blood flow to the liver is well-oxygenated blood provided by the hepatic artery and the other 70%, provided by the portal vein, is the poorly oxygenated blood venous outflow from the spleen and gastrointestinal tract<sup>7</sup>. Liver diseases such as fatty liver, shock, or tumor change the blood flow in the hepatic microcirculation. To determine tissue oxygenation, one needs to measure both the blood flow and oxygen concentration in the microcirculation. The lack of commercially available instruments for making these measurements in the hepatic microcirculation has made it hard to obtain physiological data in this field.

An important point in animal preparation steps is the fixation of the liver on a plastic plate. Loose fixation results in liver movement synchronized with respiration, which interferes with microscopic observation and makes analysis difficult. Tight fixation, however, causes blood flow stasis. Blood flow conditions should be observed through a low-power microscope during the operative procedure. High-power lasers easily injure microvessels, so the frequency of the irradiation should be minimized. 1 Hz is usually enough for temporal measurement<sup>8</sup>. However, a single measurement of pO<sub>2</sub> takes less than 1 ms, which means that the repetition frequency can be increased to 1 kHz for rapid pO<sub>2</sub> change, as necessary. Furthermore, the photochemical reaction of Pd-TCPP generates singlet oxygen that damages microvessels and induces platelet aggregation<sup>9</sup>. The combination of fluorescence imaging and pO<sub>2</sub> measurement can measure blood flow dynamics and oxygen concentration all together. The mercury lamp irradiation exciting FITC also excites Pd-TCPP, however, thereby generating much singlet oxygen and leading to blood flow stasis. When both blood flow and oxygen tension are measured in the same animal, the FITC imaging should be done before Pd-TCPP is administered. Additionally, systemic oxygen level is indicative of the oxygen consumption in the tissue. It is preferable to perform respiratory management during experiments or analyze blood gas levels after experiments.

We used APAP to induce acute hepatitis, in part because a high dose of APAP is known to lead to cell necrosis in the pericentral region<sup>10</sup>. Partial oxygen pressure measurement showed the 20.9-mmHg pO<sub>2</sub> difference between portal venules and central venules in control mice decreased to 16.9 mmHg in the APAP-treated mice and that the pO<sub>2</sub> was higher throughout the hepatic microcirculation. The oxygen gradient in the hepatic microcirculation is mainly the result of oxygen supplied from portal venules being consumed in hepatocytes. The higher dose of APAP induces necrosis in the pericentral region<sup>11, 12</sup>, and which could decrease oxygen consumption downstream as shown in **Figure 3c**. To determine detailed mechanisms underlying liver hepatitis, especially relationship between tissue necrosis and oxygen level, quantification of the extent of necrosis, and serum levels of bilirubin, ALT, AST, and inflammatory cytokines need to be measured. A lot of liver diseases are related to hepatic microcirculation, in other words, to the blood flow distribution, oxygen delivery, and oxygen consumption in liver tissue. In cases of chronic alcohol intake, about 10–15 % of alcohol is metabolized by the microsomal ethanol-oxidizing system, and the alcohol-induced molecule P450IIE1 generates NAPQI<sup>13</sup>, a toxic metabolite that leads to pericentral hepatocyte necrosis<sup>14</sup>.

In conclusion, our optical measurement method can be used to investigate physiological and pathological mechanisms by analyzing microvascular structure, diameter, blood flow velocity, and oxygen tension.

## Disclosures

We have nothing to disclose.

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