Validation of OPS imaging for microvascular measurements during isovolumic hemodilution and low hematocrits

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Harris, A. G., I. Sinitsina, and K. Messmer. Validation of OPS imaging for microvascular measurements during isovolumic hemodilution and low hematocrits. Am J Physiol Heart Circ Physiol 282: H1502–H1509, 2002; 10.1152/ajpheart.00475.2001.—Orthogonal polarization spectral (OPS) imaging is a new technique that can be used to visualize the microcirculation with reflected light. It uses hemoglobin absorption to visualize the red blood cells (RBCs). Thus the method could fail at low hematocrit (Hct). The aim of this study was to validate OPS imaging for quantitative measurements of diameter and functional capillary density (FCD) under conditions of hemodilution of varying degrees to achieve a wide range of Hcts. The validation was performed in the dorsal skinfold chamber of nine awake Syrian golden hamsters. Measurements of vessel diameter and FCD were performed off-line using Cap-Image on video sequences captured using OPS imaging and standard intravital fluorescence microscopy at baseline, 85, 70, 55, and 40% of the initial Hct. For hemodilution, isovolumic exchange of blood for 6% Dextran 60 was performed. Bland-Altman plots for the vessel diameter and FCD show good agreement between the two methods for both parameters at all studied Hcts. As expected, there was a systematic bias of about 4 μm in the diameter measurements since the RBC column was measured and not the intravascular diameter. In conclusion, OPS imaging can be used to measure diameter and FCD at a wide range of Hcts.

Cytoscan; orthogonal polarization spectral imaging

In recent studies it was shown that diameter and functional capillary density (FCD) could be quantitatively measured using the orthogonal polarization spectral (OPS) imaging technology incorporated into the Cytoscan E-II (Cytometrics, Philadelphia, PA) under both physiological and pathophysiological conditions (6, 9). These studies were carried out in the dorsal skinfold chamber of the awake Syrian golden hamster and were performed by comparing the measurements made using intravital fluorescence microscopy images with those made using Cytoscan E-II images.

The Cytoscan E-II images red blood cells (RBCs) through the absorbance of hemoglobin (6). The measurement of diameter is dependent on good visualization of the RBC column in the vessel under study. The measurement of FCD requires the observation of RBCs moving through the capillaries. Because the measurement of both of these parameters requires the presence of RBCs, their measurement could be disturbed or influenced during hemodilution. A decrease in the number of RBCs in the venules could lead to an incomplete filling of the vessel, which in turn could result in an underestimation of the vessel diameter. For the measurement of FCD, a decreased number of circulating RBCs could cause too few perfused capillaries to be counted and therefore an underestimation of the FCD.

The correct measurement of the vessel diameter is also of critical importance for the noninvasive determination of hemoglobin and hematocrit using the OPS imaging technology incorporated into the Hemoscan (Cytometrics). At low hemoglobin concentrations, where a proper measurement of the hemoglobin concentration itself becomes very important, an underestimation of the diameter could cause the measured hemoglobin value to appear much greater than it actually is, thereby compromising clinical assessment of the patient.

The aim of the present study was therefore to validate the use of the Cytoscan E-II for measuring vessel diameter and FCD during isovolumic hemodilution against standard intravital fluorescence videomicroscopy (IVM).

Materials and Methods

Animal Model and Preparation

Nine male Syrian golden hamsters (40–60 g body wt) were obtained from Charles River Wiga (Sulzbach, Germany). They were kept at 21°C in a normal 12:12-h light-dark cycle and fed laboratory food (120 mg vitamin E, 18,000 IU vitamin A/kg) ad libitum (ssniff, Spezialdiäten, Soest, Germany). They had free access to drinking water. Before the experimental procedure, they were housed in groups of two or three hamsters, and after the implantation of the chamber, they were then caged individually. The experimental procedures have previously been approved by the local ethical committee.

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The dorsal skinfold chamber in awake male Syrian golden hamsters was the model used for the study. It is a highly standardized and controlled model that has been used previously for isovolumic hemodilution studies (12–14). At the time of the experiments, the animals were 6–8 wk old and had a body weight of 60–80 g.

**Surgical procedure.** The titanium chambers (machine shop, Institute for Surgical Research) and the surgical implantation procedure used in this study are based on that of Endrich et al. (4) with only a few minor modifications. Briefly, the hamsters were anesthetized by intraperitoneal injection of ketamine-xylazine [100 mg/kg body wt (Ketavet, 50 mg/ml, Parke-Davis, Berlin, Germany); 10 mg/kg body wt (Rompun, 2%, Bayer, Leverkusen, Germany)]. The hair was removed from the back of the animals, and an extended double layer of skin on the dorsal skinfold was sandwiched between two symmetrical titanium frames. A circular area of 15 mm in diameter from one area of skin was completely removed, and the remaining layers (consisting of the epidermis, subcutaneous tissue, and thin striated skin muscle) were covered with a glass coverslip incorporated into one of the frames. After a recovery period of 24–48 h, five polyethylene catheters were inserted into the jugular vein and the carotid artery under the same anesthesia protocol. The catheters were passed subcutaneously to the dorsal side of the neck and secured to the titanium frames. The animals tolerated the dorsal skinfold chambers well, and no effect on the eating and sleeping habits were observed. The animals were allowed a recovery period of 24–48 h before the experimental observations were made to eliminate the effects of anesthesia and surgical trauma on the microvasculature.

**Intravital microscopy.** The setup and microscope used to make the standard fluorescent videomicroscopy measurements have been described in detail by Harris et al. (7). For the experiments, the awake animals were immobilized in a Plexiglas tube and the chamber was attached to a microscope stage, which was computer controlled to allow for repeated scanning of identical segments of microvessels. A 10-fold water immersion objective (Zeiss Axioshot vario 100 HD microscope, Acrophan ×10/0.5 W, Zeiss, Oberkochen, Germany; total magnification 264-fold) was used to observe the microvasculature under epi-illumination. The fluorescent images were captured using a charge-coupled device (CCD) videocamera (FK 6990 IQ-S, Piper, Schwerte, Germany). The images were recorded onto S-VHS videotape (videorecorder SVO-9500 MDP, Sony, Kln, Germany) for later off-line analysis. For transillumination, a 15-V, 150-W halogen lamp (Schott 1500 electronic, Schott, Mainz, Germany) was used in combination with a green filter (wavelength 546’/12, Zeiss) to enhance contrast. Epi-illumination was achieved using a 12-V, 100-W halogen lamp (Zeiss). The halogen lamp was used in conjunction with the Zeiss filter set 09 (BP 450–490, PT 510, LP 520) for measurements involving fluorescein isothiocyanate-labeled dextran (FITC-dextran).

To take advantage of the computer-controlled microscope functions, the Cytoscan E-II (Cytometrics) was attached to the shaft of the microscope using a specially designed C-clamp. The Plexiglas stage was modified so that there were two different pairs of holes with which the stage could be attached to the motorized plate. One pair of holes aligned the chamber under the fluorescence microscope and the other set aligned the chamber under the Cytoscan E-II. By simply moving the stage from one pair of holes to the other, the identical region of interest could be observed sequentially by either system. The Cytoscan E-II was equipped with a lens system that yielded a final magnification of 450-fold. The light source for the Cytoscan E-II is a variable halogen bulb with a maximum current of 0.73 A and a color temperature of 3,250 K (WelchAllyn, Skaneateles Falls, NY). To easily visualize erythrocytes, the polarized light is passed through a green filter. The Cytoscan E-II images were captured using a mini-CCD camera (Costar CV-M536 CCIR, JAI, Japan) and recorded on S-VHS videotape using the same videorecorder as the IVM system.

For the measurement of venular diameter, seven regions of interest within the striated muscle in the chamber that contained one or more small venules ranging in diameter from 19–84 μm were selected. For the measurement of FCD, six regions of interest that contained only capillary beds and no large vessels were selected. The exact same vessels and capillary areas were observed with both methods. FITC-dextran (0.05 ml, 3%, M, 150,000, Sigma, St. Louis, MO) was used for contrast enhancement of the microcirculation for the fluorescence microscopy measurements.

**Microcirculation measurements.** To facilitate the use of the Cytoscan E-II, the metal ring used to hold the chamber coverslip in place was carefully removed using needle-nosed pliers. Under transillumination, the seven venular and six capillary regions of interest were randomly selected and their position stored in the computer. With the use of the computer-controlled stage and the specially designed stage, these positions can be relocated at any time for observations with either the IVM or the Cytoscan E-II. The baseline measurements were then made with both systems in a randomized order. Shortly before the fluorescence microscopy measurements, an intravenous injection of FITC-dextran was given. For the fluorescence measurements, each of the seven venular areas was observed for ~20 s followed by the observation of the six capillary beds for ~30 s each. Throughout the observations, special care was taken to minimize the amount of epi-illumination on the tissue. The total exposure time under epi-illumination was ~10 min/observation period. For the Cytoscan E-II measurements, each of the observation areas was videotaped for ~30 s.

**Isovolumic hemodilution and macrohemodynamic measurements.** After the baseline conditions were recorded, a small blood sample was drawn from the arterial catheter (0.1 ml), and the hematocrit and hemoglobin were determined using a Coulter A+T counter (Coulter, Miami, FL). An isovolumic hemodilution was then performed to four different hematocrits using a 6% Dextran 60 solution (Pharmalink, Spånga, Sweden). After the baseline hematocrit measurements, the animals’ hematocrit was reduced to 85% of the initial hematocrit and the microvascular measurements were repeated. The amount of blood that was exchanged for Dextran 60 was calculated using the following formula from Bourke and Smith (3).

\[
\text{Exchange BV} = \frac{\text{animal BV} \cdot \ln \left( \frac{\text{current Het}}{\text{desired Het}} \right)}{\ln(0.73)}
\]

where BV is the blood volume and Het is the hematocrit. Confirmation of the target hematocrit was performed by again drawing a small blood sample from the arterial catheter (0.1 ml) and measuring the hematocrit and hemoglobin values using the Coulter counter. If the target hematocrit was not reached, then an additional blood volume was exchanged if the hematocrit was too high, or a 30-min waiting period was observed to allow the spleen to release RBCs if the hematocrit was too low. Additional isovolumic hemodilutions were performed to bring the hematocrit to 70, 55, and 40% of the initial value. In addition, in seven animals, a further isovolumic hemodilution was performed to bring the animals to a hematocrit that was 25% of the initial hematocrit.
To perform the isovolumic hemodilution, the arterial and venous catheters were connected to two 5-ml Hamilton syringes (Hamilton Bonaduz, Bonaduz, Switzerland). The syringe on the arterial side was empty and heparinized, and the syringe on the venous side was filled with Dextran 60. The syringes were placed in a Harvard pump (Harvard 33 Syringe Pump, Harvard Apparatus, Holliston, MA), and the calculated blood volume was exchanged by withdrawing blood from the arterial catheter and infusing Dextran 60 through the venous catheter at a rate of 0.12 ml/min. Throughout the experiments, the mean arterial pressure and heart rate were monitored to ensure that a true isovolumic hemodilution was being performed.

Microcirculatory analysis. The images from both systems were analyzed during playback using the computer-assisted microcirculation analysis system (Cap-Image) (Dr. Zeintl, Heidelberg, Germany) (11). This system uses pixel size to make microvascular measurements. The system is first calibrated to determine the pixel size using a recording of a micrometer in both a horizontal and vertical orientation. The diameter can then be calculated by summing up the length of the pixels that compose a line drawn across the vessel by the operator. For the FCD calculation, the operator is required to trace out all of the RBC perfused capillaries. A perfused capillary is defined as a capillary that has at least one RBC moving through it during a 30-s observation period. The operator traces out the path of the moving RBCs within the capillaries. With the sum of the length of the pixels that make up the marked vessels, the length of the perfused capillaries can be determined. The area of interest in which the measurement is made can also be calculated from the pixel size. Dividing the length by the area gives the FCD. The vessel diameter and FCD were determined from both the FITC images and the Cytoscan E-II images. FCD is assessed by Cap-Image as the length of RBC-perfused capillaries per observation area (in cm²). To compensate for the different magnifications between the two methods, the FCD from the FITC images was determined using an observation window with the same area as that of the Cytoscan E-II images.

Statistical Analysis

The macrohemodynamic parameters were analyzed using a Friedman repeated-measures ANOVA on ranks followed by an all pairwise multiple comparison procedure (Dunn’s method). The microcirculatory parameters were also analyzed using these tests for differences at the different hematocrits. The level of significance was set at \( P < 0.05 \). The microcirculatory data were analyzed using Bland-Altman plots that are the best statistical method for assessing agreement between different methods where neither method yields the true value (1, 2). The data are plotted as a scatter plot of the mean value of the two methods vs. the difference between the two methods.
Fig. 1. Bland-Altman plots of the diameter measurements at baseline (top left; n = 90), 85% of the original hematocrit (Hct) (top right; n = 70), 70% (middle left; n = 90), 55% (middle right; n = 90), 40% (bottom left; n = 90), and 25% (bottom right; n = 70). For each Hct, the mean difference is nearly zero, and <5% of all of the measurements lie outside 2 SD of the percent difference, which represents a 95% confidence interval in the measurement. The solid line represents the mean difference or the estimated bias. Because it is almost zero, this indicates that there is no systematic difference between the two methods. The dotted line represents a linear fit to the data points, and it has a slope of almost zero, indicating that the variance in the measurement is constant throughout the entire diameter range. The plots therefore show good agreement between both methods at all 6 Hcts. IVM, intravital fluorescence videomicroscopy.
USE OF THE CYTOSCAN DURING HEMODILUTION

Functional Capillary Density (FCD) (Baseline)

Functional Capillary Density (FCD) (85% HCT)

Functional Capillary Density (FCD) (70% HCT)

Functional Capillary Density (FCD) (55% HCT)

Functional Capillary Density (FCD) (40% HCT)

Functional Capillary Density (FCD) (25% HCT)
the initial value without any significant changes in the macrohemodynamics. At 40% of the initial hematocrit, a critical value has been reached where the blood has been thinned to the point that the remaining RBCs are no longer capable of transporting enough oxygen to meet the needs of the tissue. This results in a drop in mean arterial pressure, and in an effort to compensate for this, the animal’s heart rate increases.

At a hematocrit of 25% of the initial value, tissue damage begins to occur and the image quality decreases due to edema and swelling of the tissue. At this point, there is also a significant decrease in the FCD. Otherwise, both parameters remained constant over the entire range of hematocrits studied, indicating the reproducibility of the measurements at a wide range of hematocrits. The values measured here are also comparable with those of previous studies under physiological conditions (6, 9) as well as the control values obtained from interventional studies (8, 15). The drop of FCD seen at the very low hematocrit is likely due to the fact that there are simply too few RBCs to visualize. Because a “functional capillary” is defined as a capillary that has at least one RBC flowing through it during a 30-s observation period, at extremely low hematocrits the number of functional capillaries will decrease simply because there are too few RBCs travelling through the microcirculation (12–14). Thus the decrease in FCD is not a methodological error introduced by the low hematocrit, but rather a reflection of the actual decrease in RBC delivery to the tissue.

The fact that the FCD does not significantly decrease until the hematocrit has reached 25% of the initial value is also an indication that there are no preferential pathways for RBCs through the capillary network. If there were preferential pathways, one would expect the FCD to drop significantly at a much higher hematocrit as RBCs are diverted to the preferential pathways.

The mean ± SD values for both parameters with the two different techniques are comparable at all hematocrits (Table 2). There is very good agreement between the two for the FCD measurements. In the diameter measurements, the Cytoscan E-II measurements are less than that of the fluorescence microscopy measurements, but the relative changes that occur within the two systems are comparable.

From the Bland-Altman plots, a true comparison between the two systems can be easily made. The measurement of FCD shows very good agreement between the two methods at all hematocrits. For each hematocrit, the mean difference is nearly zero, and <5% of all of the measurements lie outside 2 SD of the percentage difference, the criterion discussed by Bland and Altman (1), which represents a 95% confidence interval in the measurement. The solid line represents the mean difference or the estimated bias (2). Because it is almost zero, this indicates that there is no systematic difference between the two methods. The dotted line represents a linear fit of the data points, and it has a slope of almost zero, indicating that the variance in the measurement is constant throughout the entire diameter range. The plots therefore show good agreement between both methods at all Hct.

**Fig. 2.** Bland-Altman plots of the functional capillary density (FCD) measurements at baseline (top left; n = 54), 85% of the original Hct (top right; n = 42), 70% (middle left; n = 54), 55% (middle right; n = 54), 40% (bottom left; n = 54), and 25% (bottom right; n = 42). For each Hct, the mean difference is nearly zero, and <5% of all of the measurements lie outside 2 SD of the percent difference, which represents a 95% confidence interval in the measurement. The solid line represents the mean difference or the estimated bias. Because it is almost zero, this indicates that there is no systematic difference between the two methods. The dotted line represents a linear fit of the data points, and it has a slope of almost zero, indicating that the variance in the measurement is constant throughout the entire diameter range. The plots therefore show good agreement between both methods at all Hct.

AJP-Heart Circ Physiol • VOL. 282 • APRIL 2002 • www.ajpheart.org
variance in the measurements. The level of accuracy as indicated by the comparison of the SD values is also acceptable. However, in the diameter measurements there is a systematic bias that occurs since the mean difference is not zero. The Cytoscan E-II measures diameters that are ~4 μm less than those from fluorescence microscopy, a result comparable to an earlier study performed with this instrument during ischemia-reperfusion (9). As previously discussed, this result is actually to be expected given the nature of the measurements with fluorescence microscopy (9). Gretz and Duling (5) showed that interfacial scattering causes an overestimation of capillary diameters by as much as 10%. In this calibration study, microspheres of a known diameter were introduced into the microcirculation. The diameters were then measured using a variety of focusing techniques. The study showed that when proper care is taken it is possible to accurately measure vessel diameter. However, a few sources of error, such as interfacial scattering, cannot be eliminated. The interfacial scattering occurs as a result of the refractive index mismatch between the tissue and the plasma, which leads to the out-of-focus halo of light making the object appear larger due to the acceptance angle of the objective.

As was also discussed by Harris et al. (9), the two methods are actually measuring two different quantities. In the IVM measurements, FITC-dextran is used as a plasma marker. The diameter of the vessel is then measured as the distance within the vessel between the two edges of the fluorescence, which is the distance between two endothelial cells on opposing sides of the vessel. With the Cytoscan E-II, the diameter is measured as the width of the RBC column flowing through the vessel. It is well known that between the RBCs and the endothelial cell there is a layer of plasma up to 1 μm thick (10). Added on to this discrepancy is the further overestimation of the diameter due to interfacial scattering. Therefore, it is to be expected that the Cytoscan E-II diameter measurements should be less than that of the fluorescence measurements, and hence the systematic difference observed in both the venular and arteriolar diameter measurements is explained. This difference is also constant over the entire range of hematocrits studied, indicating that the measurement of diameter with the Cytoscan E-II is accurate and reproducible over the entire range.

It should be noted, however, how the measurement of diameter was made at the lower hematocrits from images collected with the Cytoscan E-II. At the normal hematocrit, the RBC column is tightly packed and really is a column with two distinct and smooth edges. As the hematocrit decreases, this smooth boundary disappears and becomes pitted with gaps and holes on the edge. At the very low hematocrits, there are more gaps than RBCs, and the measurement of diameter becomes more difficult, especially on a single image. However, because a video sequence is recorded, the diameter can be measured by integrating a series of images. In this manner, the diameter of a certain cross section of the vessel is determined by measuring the outer edge of the “red cell column,” which is determined by locating the outermost RBCs that pass through the cross section during the observation period. Individual measurements of diameter for a given image are all likely to be less than the dynamic measurement made using the two extremes as edges of the red cell column. Thus, although the manual measurement made with the eye yields a reproducible value over a wide range of hematocrits, automated methods that look only at individual frames might measure reduced diameters with decreasing hematocrits.

Conclusions

The new and unique technology of the Cytoscan E-II produces images that can be used to make accurate and reproducible quantitative measurements of diameter and FCD at not only normal, but also at very low hematocrits. However, at the low hematocrits, the measurements become more difficult since a larger number of individual images must be analyzed to make accurate measurements. Thus caution is needed when these measurements are automated. However, the measurements can accurately be made. Therefore, the Cytoscan E-II should prove to be a valuable new tool for the study of the microcirculation in solid organs in a variety of clinical settings, even when the hematocrit is extremely low.

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