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The Cytoscan™ Model E-II, a New Reflectance Microscope for Intravital Microscopy: Comparison with the Standard Fluorescence Method

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Key Words

Microcirculation · Hemodynamics · Diameter · Intravital microscopy · Instrumentation

Abstract

The Cytoscan™ Model E-II (Cytometrics Inc., Philadelphia, Pa., USA) is a newly developed instrument which functions as an intravital microscope and is small and easily portable. Through the use of orthogonal polarization spectral (OPS) imaging, the Cytoscan Model E-II delivers images of the microcirculation which are comparable to those achieved with intravital fluorescence videomicroscopy (IFM), but without the use of fluorescent dyes. The purpose of this study was to validate the Cytoscan Model E-II instrument against IFM. The experiments were carried out on striated muscle in the dorsal skinfold chamber of the awake Syrian hamster. The following parameters were measured in identical regions of interest in the same animal under baseline conditions and 0.5 and 2 h after a 4-hour period of pressure-induced ischemia: arteriolar diameter, venular diameter and venular red blood cell velocity. Bland-Altman plots showed good agreement between the two techniques for venular red blood cell velocity. As expected, arteriolar and venular diameters as measured by the Cytoscan were on average 5 μm smaller than the values from IFM, since the Cytoscan measures the red blood cell column width and IFM measures luminal diameter. Thus, OPS imaging can be used to make valid measurements of microvascular diameter and red blood cell velocity in tissues.

Introduction

Intravital microscopy has been used for many years to make quantitative observations of the microcirculation. Early experiments were limited to very thin tissues such as the bat wing [1] or tadpole tail [2], in order to ensure that enough light penetrated the tissue by transillumination so that measurements could be made.

More recently, the use of fluorescent dyes with epiillumination has become widespread because it offers several advantages. The image contrast is greatly improved and it is also possible to selectively mark a specific type of cell, making their identification easier and more quantitative. Because of the high image contrast achieved when using fluorochromes, intravital microscopy could be performed on more solid tissues such as the liver and brain.

However, the use of fluorescent dyes also has several disadvantages. The binding of the dye to the cell could have undesired side effects such as altering the cell function or even killing the cell through some toxicity reaction.

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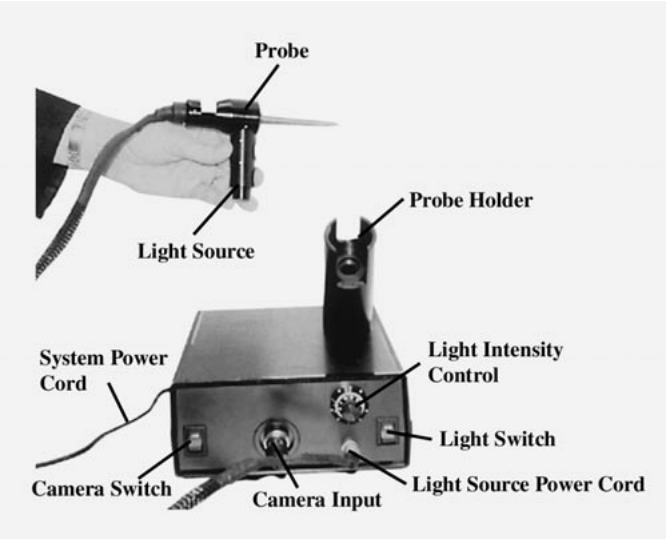

Fig. 1. The Cytoscan Model E-II instrument. Note the small size and portability of the instrument compared to standard intravital microscopes.

The presence of a fluorochrome and the high light intensities used in epiillumination can themselves lead to so-called phototoxic effects [3–5]. Phototoxic effects result from the high energy levels present in the tissue and can lead to the production of reactive oxygen species. Their release can cause leukocyte activation, platelet aggregation, vasoconstriction and a loss of capillary perfusion. In this respect, the measurement itself can alter the very parameters that the researcher is trying to measure.

In addition, studies of the microcirculation are restricted almost exclusively to animal models because of the necessity of the use of fluorescent dyes. They are in general themselves toxic and there is only a single dye which has been approved for use in humans. Because of the risk of phototoxic effects, it is used very sparingly. Measurements of the human microcirculation can be made through the capillary nailfold bed without the use of fluorescent dyes, but this procedure has only limited application and has yet to find widespread clinical acceptance [6]. Since the microcirculation plays a critical role in many disease states, such as diabetes and hypertension, a knowledge of its structure and function in humans is of great importance [6]. Thus, the ability to study the microcirculation of solid organs without the use of fluorescent dyes would represent important progress for clinical medicine.

The Cytoscan™ Model E-II (Cytometrics Inc., Philadelphia, Pa., USA; Cytoscan is a trademark of Cytometrics Inc.), shown in figure 1, is a newly developed, small, hand-held device. Because of its small size, it can easily be transported and used in a variety of situations which would be impossible to access with a large, standard intravital microscope. The Cytoscan Model E-II makes use of orthogonal polarization spectral (OPS) imaging to obtain images in reflected light using hemoglobin absorbance, which can be analyzed quantitatively off-line [7]. Slaaf et al. [8] have shown that the use of two polarizers could improve image contrast during epiillumination with white light in a standard microscope. With OPS imaging, high-contrast 'transillumination-like' images can be obtained not only from thin tissues, but from the surface of large solid organs as well. Given the problems associated with fluorescent dyes, as discussed above, such a device could be a valuable new tool for studying the microcirculation, particularly in humans. Since the microcirculation plays a central role in many cardiovascular diseases, there is a need for such a device [6]. Prior to performing studies in humans, it is first necessary to validate the device against a standard technique used to observe the microcirculation.

Two important microvascular parameters are vessel diameter and red blood cell velocity. Since OPS imaging uses hemoglobin absorbance to achieve contrast, the diameter which it measures will be that of the red cell column. In intravital fluorescence microscopy (IFM), a fluorescent dye is used to achieve contrast, so the diameter measured using this technique will be the luminal diameter, which will be larger than the diameter of the red cell column. The validation of the new method by comparison to an established one is further complicated here by the dynamics of the microcirculation. It is known that both vessel diameter and red blood cell velocity are dynamic parameters which change over time. Since the vessels cannot be imaged with the two techniques simultaneously, the measurements which are made on the exact same vessel are separated in time. This means that the true values to be measured are actually different for the two techniques. Therefore, a large amount of scatter is to be expected within the data when directly comparing the two techniques. Despite these complications, validation of OPS imaging is still necessary to show that it can be used to make quantitative measurements of microvascular parameters.

Therefore, the aim of this study was to validate the use of the Cytoscan Model E-II for making microvascular measurements against standard IFM under normal conditions and in a disease state, in this case after ischemia/reperfusion injury.

Materials and Methods

The dorsal skinfold chamber in 10 awake male Syrian golden hamsters was the model used to make the comparative microvascular measurements. It is a highly standardized and controlled model which has been used extensively to study ischemia/reperfusion injury [9–11]. The animals were purchased from Charles River Wiga (Sulzbach, Germany); they were 6- to 8-week-old and had a body weight of 60–80 g. They were kept at 21 °C in a normal cycle of 12 h of light and 12 h of darkness, and fed laboratory chow (120 mg of vitamin E, 18,000 IU of vitamin A per kilogram) ad libitum (ssniff, Spezialdiäten GmbH, Soest, Germany).

Surgical Procedure

The chambers and the surgical implantation procedure used in this study are based on those presented by Harris et al. [11] and Endrich et al. [12], with only a few minor modifications. Briefly, the hamsters were anesthetized by intraperitoneal injection of ketamine/xylazine (100 mg/kg body weight; Ketavet, 50 mg/ml; Parke-Davis, Berlin, Germany)/(10 mg/kg body weight; 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. From one of the skin layers, a circular area 15 mm in diameter was completely removed and the remaining layers (consisting of the epidermis, subcutaneous tissue and a thin striated skin muscle) were covered with a glass coverslip incorporated into one of the frames.

After a recovery period of 24–48 h, a fine polyethylene catheter was inserted into the jugular vein under the same anesthesia protocol for the application of fluorescent dye. The catheter was passed subcutaneously into the dorsal side of the neck and secured to the titanium frames. The animals tolerated the dorsal skinfold chambers well and no effects on their eating and sleeping habits were observed. The animals were allowed another recovery period of 24–48 h before 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 IFM measurements have been presented in detail by Harris et al. [13]. The awake animals were immobilized in a plexiglass tube and the chamber was attached to a microscope stage which is computer controlled to allow for repeated scanning of identical segments of microvessels. A 10-fold water immersion objective (Zeiss Axiotech vario 100 HD microscope, Acroplan 10×/0.5 W, Zeiss, Oberkochen, Germany; pixel resolution 1.5 μm) was used to observe the skin microvasculature under epiillumination. The fluorescent images were captured using a charge-coupled device video camera (FK 6990 IQ-S, camera size 2 cm, Piper, Schwerte, Germany). The images were recorded onto S-VHS video tape (video recorder SVO-9500 MDP, Sony, Köln, Germany) for later off-line analysis. For transillumination, a 15-volt, 150-watt halogen lamp (Schott 1500 electronic, Schott, Mainz, Germany) was used in combination with a green filter (wavelength 546 ± 12 nm; Zeiss) to enhance contrast. Epiillumination was achieved using a 12-volt, 100-watt halogen lamp (Zeiss). The halogen lamp was used in conjunction with the Zeiss filter set 09 (BP 450–490, FT 510, LP 520) for measurements involving fluorescein isothiocyanate-labeled dextran (FITC-dextran).

To take advantage of the computer-controlled functions of the X-Y plate, the Cytoscan Model E-II was attached to the shaft of the microscope using a specially designed C clamp. The stage was modified so that there were two different pairs of holes through which the stage could be attached to the motorized plate. One pair of holes aligned the chamber under the fluorescence microscope, the other set aligned the chamber under the Cytoscan Model E-II. By simply moving the stage from one pair of holes to the other, the identical region of interest could be observed with the two systems. The Cytoscan Model E-II was equipped with a 10× lens system with a numerical aperture of 0.26, yielding a pixel resolution of 1 μm. The Cytoscan Model E-II images were captured using a mini-CCD camera (Costar CV-M536 CCIR, camera size 0.5 inches, JAI Corporation, Japan) and recorded on S-VHS video tape using the same video recorder as the IFM system. The light source for the Cytoscan Model E-II is a variable halogen bulb with a maximum current of 0.73 A and a color temperature of 3,250 K (Welch Allyn, Skaneateles Falls, N.Y., USA). In order to easily visualize erythrocytes, the polarized light is passed through a green filter to illuminate the tissue with light at a wavelength of 550 nm.

For the measurement of arteriolar diameter, two regions of interest containing terminal arterioles ranging in diameter from 20 to 85 μm were selected. For the measurement of venular diameter and venular erythrocyte velocity, seven regions of interest within the striated muscle in the chamber which contained one or more small venules ranging in diameter from 15 to 65 μm were selected. FITC-dextran (0.05 ml, 3%, M_r = 150,000; Sigma, St. Louis, Mo., USA) was used for contrast enhancement of the microcirculation for the IFM measurements.

Microcirculation Measurements

Under transillumination, the two arteriolar regions and the seven venular regions of interest were randomly selected and their position stored in the computer. Using the computer-controlled plate and the specially designed stage, these positions can be relocated at any time for observations either by IFM or the Cytoscan Model E-II. The baseline measurements were then made with both systems in a randomized order, so that in half of the animals, the IFM measurements were made first and in the other half, the Cytoscan measurements were made first. Shortly prior to the fluorescence microscopy measurements, an intravenous injection of FITC-dextran at the aforementioned dose was given. For the fluorescence measurements, each of the seven venular areas and two arteriolar regions were observed for approximately 20 s. Throughout the observations, special care was taken to minimize the amount of epiillumination on the tissue. The total exposure time under epiillumination was less than10 min per observation period. For the Cytoscan Model E-II measurements, each of the observation areas was videotaped for approximately 30 s.

Ischemia Induction

To validate the Cytoscan Model E-II measurements in a complex pathological situation, the chamber tissue was subjected to a 4-hour period of pressure-induced ischemia following the recording of the baseline conditions [14]. The ischemia was induced by gently pressing the skin tissue against the cover slip from the backside with a silicone pad by slowly tightening an adjustable screw under microscopic control until the blood vessels emptied and the tissue appeared white. Following the 4-hour ischemia, the screw was released and the silicone pad was removed. The observation procedure was repeated after 0.5 and 2 h of reperfusion. In five animals, the fluorescence microscopy measurements were made first, and in the other

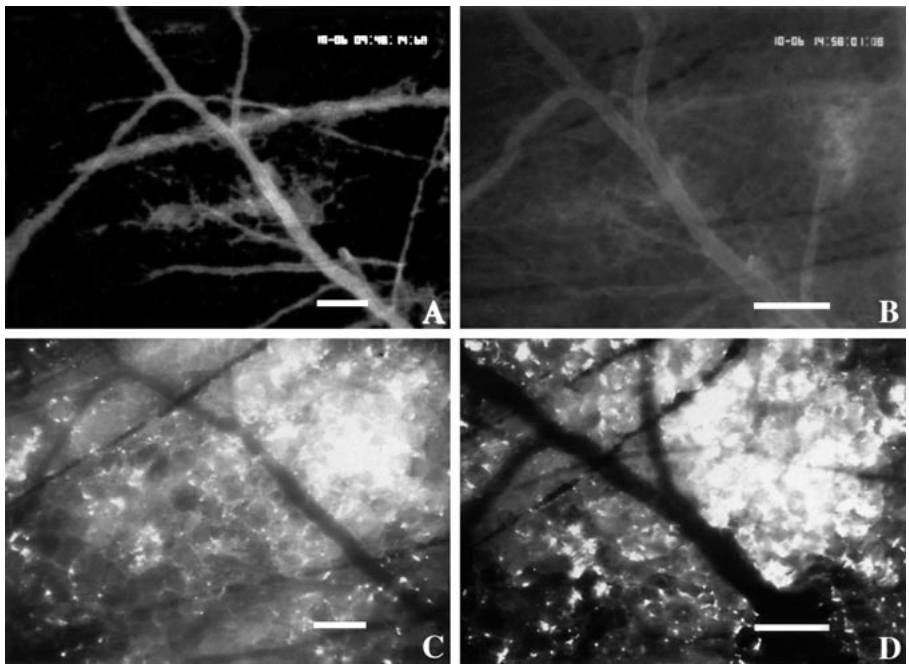

Fig. 2. Typical images from standard fluorescence microscopy (**A**) and the Cytoscan Model E-II (**C**) under baseline conditions. Pictures of the same venule following a 4-hour period of pressure-induced ischemia and 0.5 h of reperfusion from standard fluorescence microscopy (**B**) and the Cytoscan Model E-II (**D**) are also shown.

five animals, the Cytoscan Model E-II measurements were made first, to assure that no bias was introduced as a result of the measurements being made consecutively and not simultaneously. At the conclusion of the study, the animals were sacrificed with an overdose of pentobarbital sodium (Narcoren®, Rhone Merieux GmbH, Laupheim, Germany).

Microcirculatory Analysis

The images from both systems were analyzed during playback using a computer-assisted microcirculation analysis system (Cap-Image; Dr. Zeintl, Heidelberg, Germany) [15]. The system was calibrated for the two devices according to the manufacturer's instructions. The vessel diameter and midstream red cell velocity were determined from both the FITC images and the Cytoscan Model E-II images. The vessel diameter was measured with Cap-Image using the manual mode, where the user determines the diameter with two mouse clicks on the outer edges of the contrast-producing structure. For the IFM measurements, the diameter was measured from the two edges of the plasma fluorescence (inner vessel wall). For the Cytoscan measurements, the diameter was measured from the two edges of the observed red cell column (the inner vessel wall is not clearly depicted).

Velocity was determined using the line shift diagram option in Cap-Image. For this procedure, a line is drawn down the middle of the vessel. The pixels under this line are then stored for 10 s and placed vertically next to each other. Particles traveling along the line produce stripes in the vertical pixel presentation. From the length and slope of these stripes, the velocity of the particles can be calculated.

Statistical Analysis

The mean values from the average value of each experiment for each parameter were compared using a Student's t test. The data were analyzed using a linear regression and a Spearman rank order correlation (Sigma Stat 2.0, Jandel Scientific, Erkrath, Germany). The data were further analyzed using Bland-Altman plots as the best statistical method for assessing agreement between two different methods where neither method yields the true value [16, 17]. The data are plotted as a scatter plot of the mean value of the two methods versus the difference between the two methods.

Results

Typical images from fluorescence microscopy and the Cytoscan Model E-II under baseline conditions and after the ischemic insult are shown in figure 2. A total of 10 experiments in 10 different animals were carried out for this study. In each experiment, nine venules and two arterioles were examined. Table 1 shows the mean values and standard deviations for the two different techniques for the three different time points. There were no significant differences between the two techniques for any of the parameters.

The regression analyses of the data for the arteriolar diameter, venular diameter and venular red blood cell velocity were performed and the R^2 values were 0.63, 0.68 and 0.56, respectively. The Spearman rank order correlation for all three parameters was also significant, with correlation coefficients of 0.793, 0.838 and 0.745, respectively, indicating that the two methods yield comparable results.

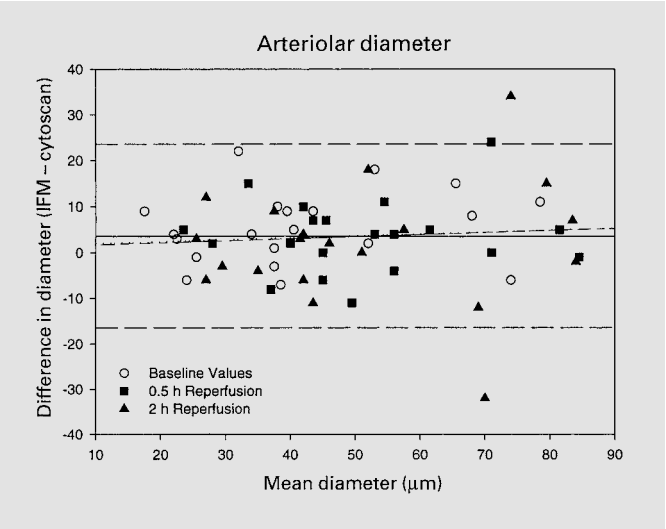

Fig. 3. A Bland-Altman plot of the arteriolar diameter measurements for all three time points (n = 60). The solid line represents the mean difference, which is 5.0 μm, and the standard deviation of the difference is 11.0 μm. The dashed lines represent ±2 standard deviations of the mean difference. The dotted line represents a linear fit of the plotted points. The two methods show good agreement despite the fact that the Cytoscan Model E-II measurements are approximately 5 μm less than the IFM measurements.

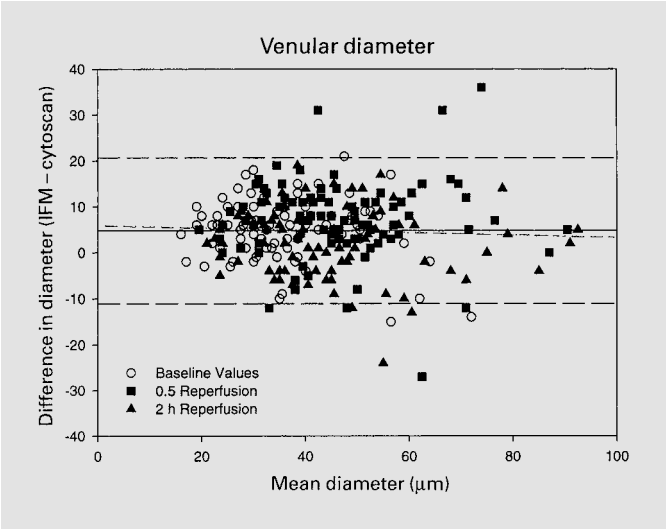

Fig. 4. A Bland-Altman plot of the venular diameter measurements for all three time points (n = 270). The solid line represents the mean difference, which is 4.2 μm, and the standard deviation of the difference is 8.7 μm. The dashed lines represent ±2 standard deviations of the mean difference (the 95% confidence interval). The dotted line represents a linear fit of the plotted points. The two methods show good agreement despite the fact that the Cytoscan Model E-II measurements are approximately 4 μm less than the IFM measurements.

Table 1. Measured parameters of identical microvessels, given as the mean ± standard deviation (n = 10 animals)

	Baseline		Reperfusion (0.5 h)		Reperfusion (2 h)	
	IFM	Cytoscan Model E-II	IFM	Cytoscan Model E-II	IFM	Cytoscan Model E-II
Arteriolar diameter, μm	44.9 ± 15.2	40.0 ± 15.1	52.9 ± 12.7	49.3 ± 12.3	51.8 ± 17.0	50.0 ± 12.5
Venular diameter, μm	38.4 ± 4.9	33.6 ± 6.3	49.7 ± 5.3	42.5 ± 5.9	45.7 ± 6.5	43.3 ± 6.2
Venular RBC velocity, mm/s	0.62 ± 0.14	0.62 ± 0.13	0.64 ± 0.29	0.59 ± 0.26	0.66 ± 0.22	0.61 ± 0.24

RBC = Red blood cell.

The Bland-Altman plots for venular diameter and venular red blood cell velocity showed a strong similarity at all three time points. The mean difference and the linear fit to the points, as well as the 95% confidence intervals, were all very similar. Therefore, the combination of the data into a summary plot is justified. In the case of the arteriolar diameter, the similarities were not as strong, but this is due to the much lower number of measurements made, and thus, the combination of the data into a summary plot is also justified. The data from all three time points were therefore combined and the summary Bland-Altman plots for arteriolar diameter, venular diameter and venular red blood cell velocity are shown in figures 3–5, respectively. The bias and precision for the parameters were 5.0 ± 11.0 μm, 4.2 ± 8.7 μm and 0.03 ± 0.25 mm/s.

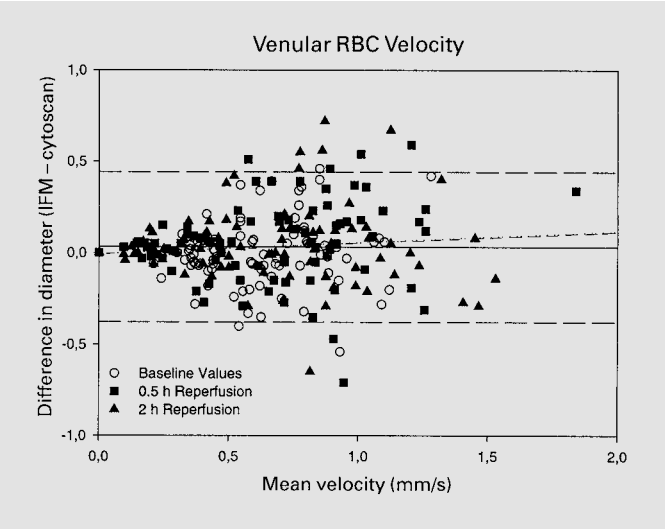

Fig. 5. A Bland-Altman plot of the venular red blood cell (RBC) velocity measurements for all three time points (n = 270). The solid line represents the mean difference, which is only 0.03 mm/s, and the standard deviation of the difference is 0.25 mm/s. The dashed lines represent ±2 standard deviations of the mean difference (the 95% confidence interval). The dotted line represents a linear fit of the plotted points. The two methods show good agreement.

Discussion

The data demonstrate that the Cytoscan Model E-II can be used to measure the three microvascular parameters studied under both physiological and pathophysiological conditions. From table 1, it can easily be seen that the mean values and standard deviations for all three parameters with the two different techniques are comparable at all time points. The regression analysis also showed a significant correlation between the two techniques for all three parameters. From the Bland-Altman analysis, it is also apparent that there is good agreement between the two techniques in respect to venular red blood cell velocity. For the diameter measurements, the Cytoscan Model E-II values are less than those from fluorescence microscopy, but the relative changes measured by the two techniques are comparable. The changes observed here were what was expected and are comparable to those observed in previous studies [11, 14].

The regression analysis and Spearman rank order correlation showed a significant correlation between the two techniques. For all three parameters, the p value was less than 0.05.

From the Bland-Altman plots, a true comparison between the two systems can be easily made. For all three parameters, the Bland-Altman plots showed good agreement between the two techniques at all three time points. This is an indication that valid microvascular measurements can be made under both physiological and pathophysiological conditions. Because there were no differences in the plots for the three separate time points, the data for each parameter were combined into one single plot.

The measurements of venular red blood cell velocity show good agreement between the two techniques, as all four criteria suggested by Bland and Altman are met: mean difference or bias of zero, slope of the linear regression of zero, 95% of all of the values within $\pm 2\sigma$, and a 95% confidence interval of an acceptable range [17]. The solid line represents the mean difference or the estimated bias [16]. Since it is almost zero, this indicates that there is no systematic difference between the two techniques. The dotted line represents a linear fit of the data points and it has a slope of almost zero, indicating that the variance between the measurements is constant throughout the velocity range studied, according to the Bland-Altman analysis. Hence, larger values do not tend to have statistically larger differences, although from the figure this does not appear to be the case. Since the variation is normally distributed, the mean difference remains constant. Less than 5% of the values lie outside of the 95% confidence interval. Furthermore, this interval is of an acceptable range. It is known that in this model using Cap-Image for the measurement of velocity, the standard deviation of repeated measurements of the same vessel over time is approximately 0.2 mm/s. This value was obtained by reanalyzing data from control animals in previous experiments. This seemingly large variability stems from the dynamic nature of the measurement. In the microcirculation, there is a large degree of heterogeneity in the flow through the small vessels, meaning that it is constantly changing even if the macrohemodynamic parameters remain constant. Thus, since the standard deviation of the difference between the two techniques (0.25 mm/s) is comparable with that of the standard deviation of the measurement with only a single method (0.2 mm/s), this range of the 95% confidence interval is acceptable. Because all four criteria set by Bland and Altman are met, it is an indication that the two methods are in agreement for the measurement of venular red blood cell velocity.

In a study recently published by De Vriese et al. [18], the authors also looked at the variability in blood flow measurements in arterioles using the line shift diagram

method in Cap-Image. Using a high-speed camera, they measured velocities up to 40 mm/s and found that repeated measurements of the same vessel differed by up to 25%, which would correspond to a standard deviation of 5–10 mm/s. This was found to be an acceptable range of variation. In the current study, a similar percentage of variation was also observed. In this context, the variability found in the current study is also acceptable.

In the measurement of microvascular diameter, both arteriolar and venular, the analysis is more complicated, due to the fact that the mean difference or bias is not zero. With the Cytoscan Model E-II, the diameter values obtained were approximately 5 μm less than those from fluorescence microscopy. This result is actually to be expected, given the nature of the measurement with fluorescence microscopy. Gretz and Duling [19] showed that interfacial scattering causes an overestimation of capillary diameter by as much as 10%. 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. They reported that diameters measured using fluorescence microscopy are overestimated by as much as 0.8 μm. Hence, scattering could account for approximately 1 μm of the bias observed in the current study.

In addition, the two methods are actually measuring two different 'diameters'. For the IFM 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 compartment, which is the distance between two endothelial cells on opposing sides of the vessel. With the Cytoscan Model E-II, the diameter is measured as the width of the red blood cell column present in the vessel. It is well known that between the red blood cells and the endothelial cell there is a layer of plasma up to 2 μm thick [20]. In fact, these two different diameters have also been discussed by Michoud et al. [21] in a study in which they examined two different algorithms for the automatic measurement of vascular diameter. The bias of 4–5 μm observed in this study can be explained by the two different diameters measured and the additional overestimation of the diameter due to fluorescent interfacial scattering. Therefore, it is to be expected that the Cytoscan Model E-II diameter values should be less than those obtained by fluorescence measurements, hence the systematic difference observed in both the arteriolar and venular diameter measurements.

Since the slope of the linear fit to the data is nearly zero in both plots, this is an indication that the bias is constant over the entire range of values studied. Thus, if the desired diameter was the distance between two opposing endothelial cells, it would be possible to correct the red cell column diameter measured using the Cytoscan to give this value. However, when extreme alterations in the local hematocrit or sludging occur, this correction factor could change.

An overwhelming majority of the values we recorded are situated within the 95% confidence interval. At first glance however, it would appear that this interval is unacceptably large due to the scatter of the data. This scatter is actually to be expected, since the vessels are not rigid tubes and are thus affected by the dynamics of blood flow. Since the measurements with the two techniques were separated by approximately 15 min, changes in vessel diameter during that time will occur. In studies in our laboratory using the same model, we found variations in the diameter of a single vessel over time of up to 8 μm, and there was a standard deviation of 4 μm using a single method. Intaglietta and Hammersen [22] have also reported that due to vasomotion, the venular vessel diameter changes on average by 10% and the diameter of small arterioles can vary by up to 100%. This greater variation in the true vessel diameter over time is reflected in our data by the larger 95% confidence interval calculated for the arterial diameter data. An additional amount of scatter is certainly also introduced by the fact that the two methods are measuring different diameters. Thus, while the scatter observed in this study for the measurement of diameter appears to be large, it is actually to be expected and therefore of an acceptable range. Since the four criteria of Bland and Altman are satisfied, it can be said that there is acceptable agreement between the two techniques for the measurement of vessel diameter. The small size and portability of the Cytoscan Model E-II is also an improvement over standard microscopes. It can easily be placed on a small cart and transported. With the handheld probe, it is also very easy to access any exposed tissue.

Conclusions

With the unique technology of the Cytoscan Model E-II, transillumination-like images of the microcirculation can be obtained using epiillumination. From these images, it is possible to make accurate off-line quantitative measurements of vessel diameter and venular red

blood cell velocity. Leukocytes can occasionally be identified, but not to an extent which allows quantitative measurements. Further improvements to the system should make the measurement of leukocytes possible. The Cytoscan Model E-II should prove to be a valuable new tool for the study of the human microcirculation, especially in solid organs where measurements without fluorescent dyes are as yet not possible. Furthermore, the ability to make measurements in the microcirculation without fluorescent dyes opens up the possibility for broad clinical applications where the use of fluorescent dyes in patients is not feasible. The small size and portability of the Cytoscan also make it possible to observe the microcirculation in situations where a standard microscope could not be used.

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