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## Articles

# Identification of Distinct Luminal Domains for Macromolecules, Erythrocytes, and Leukocytes Within Mammalian Capillaries

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## Abstract

A thick endothelial surface coat consisting of the glycocalyx and associated plasma proteins has been hypothesized to reduce functional capillary volume available for flowing plasma macromolecules and blood cells. The purpose of this study was to compare anatomic and functional capillary diameters available for macromolecules, RBCs, and WBCs in hamster cremaster muscle capillaries. Bright-field and fluorescence microscopy provided similar estimates (mean±SE) of the anatomic capillary diameter: 5.1±0.1 μm (bright field, 39 capillaries in 10 animals) and 5.1±0.2 μm (membrane dye PKH26, 18 capillaries in 2 animals). Estimates of functional diameters were obtained by measuring the width of RBCs and WBCs and the intracapillary distribution of systemically injected fluorescein isothiocyanate (FITC)-dextran 70. WBCs (5.1±0.2 μm) fully occupied the anatomic capillary cross section. In contrast, the widths of RBCs (3.9±0.2 μm, 21 capillaries in 8 animals) and FITC-dextran (4.3±0.2 μm, 21 capillaries in 8 animals) were significantly smaller than the anatomic capillary diameter. Continuous (1- to 5-minute) excitation of fluorochromes in the capillary lumen (light-dye treatment) increased the width of RBCs passing the treated site from 3.6±0.3 to 4.4±0.3 μm (6 capillaries in 4 animals) and the width of the FITC-dextran column from 4.1±0.2 to 4.6±0.3 μm (10 capillaries in 7 animals). Furthermore, light-dye treatment increased capillary tube hematocrit by 60% in 40-μm-long capillary segments compared with untreated sites in the same capillaries. It is concluded that the wall of skeletal muscle capillaries is decorated with a 0.4- to 0.5-μm-thick endothelial surface coat, which may represent the true active interface between blood and the capillary wall.

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**Key Words:** hamster cremaster muscle • functional capillary diameter • anatomic capillary diameter • bright-field microscopy • fluorescence microscopy

## ► Introduction

The surface of vascular endothelial cells is not smooth but is decorated with a matrix of extracellular proteoglycans and glycoproteins.<sup>1 2 3</sup> It has been suggested that interaction of these endothelial surface structures with components of blood on the luminal side of the vascular wall allows endothelial cells to affect capillary permeability, leukocyte-endothelial adhesion, and repair of the vessel wall.<sup>4 5 6 7</sup>

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The unknown nature of endothelial surface molecules and the fact that >90% of the volume of extracellular matrices may be composed of water<sup>8 9</sup> have complicated measurements of the true in vivo dimensions of the endothelial matrix. Electron microscopic estimates of the radial dimension of the endothelial matrix in vitro vary between 0.02 and 0.12  $\mu\text{m}$ <sup>10 11 12 13 14 15</sup> and appear to be affected by the protein content of the vessel perfusate.<sup>12 14 16</sup> However, dehydration for electron microscopy causes collapse of the polysaccharide structures,<sup>15 17</sup> suggesting that the endothelial matrix may be substantially thicker in vivo.

In fact, some studies reported thin extensions of the matrix up to 0.20 to 0.87  $\mu\text{m}$  into the lumen of blood vessels, which may be a more appropriate estimate.<sup>12 14 15</sup> Furthermore, a recent study using intravital microscopy to detect fluorescent lectins bound to specific carbohydrate sequences revealed that the endothelial matrix may be as thick as 0.45  $\mu\text{m}$  in capillaries of hamster cremaster muscle.<sup>18</sup> The anatomic diameter of those capillaries is typically  $\approx 5 \mu\text{m}$ <sup>19 20 21 22</sup>; thus, exclusion of blood components from such a matrix or restriction of plasma motion within this space would significantly reduce functional capillary volume available for blood flow. Reduction of functional capillary volume could affect oxygen supply to tissues by limiting capillary volume available for RBCs.<sup>20</sup> Furthermore, exclusion of blood from a region near the capillary wall would explain that microvascular blood flow resistance is substantially higher in vivo than estimated from in vitro experiments using narrow glass tubes.<sup>23 24</sup> Reduced functional capillary volumes will also introduce errors in estimates of capillary hydraulic permeability, which are based on the assumption that transcapillary water flow can be calculated from measurements of RBC displacement and the anatomic capillary diameter.<sup>25</sup>

The purpose of the present study was to determine if exclusion of components of blood from a region near the capillary wall could be detected in vivo. Intravital microscopy was used to observe hamster cremaster muscle capillaries at high magnification. Estimates were made of capillary diameters available for plasma macromolecules, RBCs, and leukocytes and were compared with the anatomic capillary diameter.

## ► Materials and Methods

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## Animal Preparation

Hamsters were anesthetized with intraperitoneal pentobarbital sodium (70 mg/kg body wt), and the trachea was cannulated to ensure a patent airway. The left femoral vein was cannulated for continuous infusion of 0.9% saline (0.5 mL/h) containing 10 mg/mL pentobarbital sodium. The hamster was placed on a Plexiglas platter, and the right cremaster muscle was prepared for visualization of the microcirculation as previously described.<sup>26</sup> The cremaster muscle was continuously superfused at 5 mL/min with a bicarbonate-buffered physiological salt solution containing (mmol/L) NaCl 131.9, KCl 4.6, CaCl<sub>2</sub> 2.0, MgSO<sub>4</sub> 1.2, and NaHCO<sub>3</sub> 20. The superfusion solution was gas-equilibrated with 5% CO<sub>2</sub>/95% N<sub>2</sub> to obtain a pH of 7.35 to 7.45, and the solution was maintained at 34°C. Succinylcholine (10<sup>-5</sup> mol/L, Sigma Chemical Co) was added to the superfusion solution to reduce spontaneous skeletal muscle contractions. Body temperature was maintained at 37°C to 38°C with conducted heat.

## Intravital Microscopy

Microvessels of the cremaster muscle were observed with an intravital microscope (Zeiss ACM) and a SIT video camera (model 66, Dage MTI). The hamster cremaster muscle was transilluminated with a xenon lamp (150 W) for bright-field observations or epi-illuminated with a xenon high-pressure lamp (75 W) for fluorescence microscopy. All preparations were examined at x90 (Leitz, water immersion; NA, 1.20) unless otherwise noted. A x20 objective (Leitz; NA, 0.33) acted as a long working distance condenser. Bright-field measurements were made with a 450- to 490-nm band-pass interference filter (blue light) in the light path; fluorescence measurements used an excitation filter (450 to 490 nm), a dichroic beam splitter (FT 510), and a long-pass filter (LP 520). The image was displayed on an MTI video monitor (Dage, Inc), and experiments were recorded on videotape using a Panasonic SVHS videocassette recorder for further image analysis.

## Data Analysis

Video images were imported from videotape to a Gateway personal computer (Pentium processor, 100 MHz) using Image-1 hardware and software (Universal Imaging Corp). An on-screen caliper was used for all dimensional measurements. Measurement calibration was done using an image of a 1-mm/0.01-mm stage micrometer (Graticules, Ltd).

Estimates of the anatomic capillary diameter were obtained from bright-field images as described by Gretz and Duling.<sup>22</sup> In short, the midplane of a capillary was determined by observing the changes in contrast that are associated with moving the focal plane of the microscope through the midplane of the capillary. Overfocusing of a capillary will produce an apparent light inner boundary at the capillary wall, whereas underfocusing will produce an apparent light outer boundary. By changing from overfocus to underfocus, the apparent light boundary will move from the inside to the outside of the capillary wall while moving through the midplane of the capillary. At the vessel midplane, the apparent light boundary will disappear, and only a dark wall will remain, which is to be taken as the true anatomic capillary wall. The anatomic capillary diameter was estimated by positioning digital calipers at the inside of this wall. Optical scans were made of some capillaries to obtain light-intensity profiles. These measurements confirmed that calipers were positioned at the inside of the capillary wall, where light intensity changes from dark to light (data not shown). To correct for the thickness of the caliper bars, one of the calipers was positioned on the capillary wall with its luminal surface against the capillary lumen. The other caliper was positioned in the capillary lumen with its abluminal surface

against the capillary wall. These estimates were compared with independent estimates obtained from measurements of the anatomic capillary diameter with fluorescence microscopy after capillary endothelial cells were stained with a membrane dye. Dye labeling of capillary endothelium was achieved after micropipette injection (Desjardins and Duling<sup>20</sup>) of the membrane-specific fluorescent dye PKH26 (1 to  $2 \times 10^{-6}$  mol/L, Sigma) into capillary networks. Estimates of the functional capillary diameter available for different components of the flowing blood were obtained from measurements of the width of RBCs and WBCs with bright-field microscopy and from measurements of the width of the capillary lumen occupied by FITC-dextran (molecular mass, 70 000 D) with fluorescence microscopy.

To estimate the magnitude of errors due to defocusing or image distortions, diameter measurements were also made of narrow glass tubes with dimensions similar to capillary blood vessels. Glass micropipettes of capillary dimensions were pulled on a vertical pipette puller (DKI model 700C, David Kopf Instruments) from glass tubes with an inner filament (WPI catalog No. 1B120F-4, World Precision Instruments). Narrow glass micropipettes were perfused with FITC-dextran (molecular weight, 70 000; 20 mg/mL saline) or hamster RBCs in saline (10% hematocrit) by pressurizing the micropipettes using a Pneumatic PicoPump (model PV 820, World Precision Instruments). Measurements were made of the inner glass diameter and the widths of the FITC-dextran column and of RBCs. The pipettes were immersed in immersion oil (Immersionsoel 518 C; refractive index, 1.51; Zeiss) to limit diffraction of transilluminating light at the glass surface.

### Capillary Tube Hematocrit

Estimates of capillary tube hematocrit were obtained with bright-field microscopy at x55 (Leitz; NA, 0.84) from measurements of the flux of RBCs ( $F$ ) through individual capillaries, the velocity of RBCs ( $V$ ) in each capillary, and the anatomic capillary diameter ( $D_a$ ).  $F$  was determined during slow-motion video playback by the time necessary for at least 50 RBCs to flow through a capillary.  $V$  in a capillary was determined by measuring the distance traveled over time from digitized segments of videotape. Capillary tube hematocrit ( $H$ ) was calculated from  $F$ ,  $V$ , and  $D_a$  with the following formula:

$$H = \frac{F}{V \cdot \pi / 4 \cdot D_a^2} \cdot MCV$$

where MCV ( $61 \mu\text{m}^3$ ) is mean corpuscular volume of hamster RBCs.

### Oxygen-Derived Free Radicals

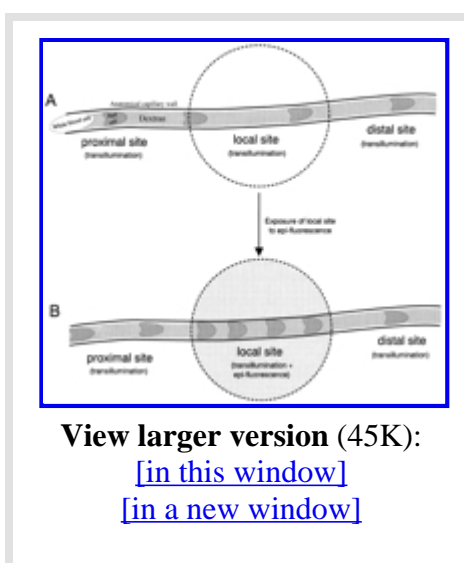
To test whether superoxide anion or hydrogen peroxide contributed to observed effects of epifluorescence on capillary tube hematocrit, the hamster cremaster vasculature was pretreated with SOD (EC 1.15.1.1, Sigma) and CAT (EC 1.11.1.6, Sigma) in a number of experiments. SOD and CAT are enzymes that catalyze the dismutation of superoxide anion and decomposition of hydrogen peroxide, respectively. Both SOD and CAT were administered as a bolus (SOD, 250 U/0.1 mL saline; CAT, 250 U/0.1 mL saline), as a continuous infusion (SOD, 29 U/min; CAT, 7.5 U/min), and in the salt solution superfusing the cremaster muscle (SOD, 50 U/mL; CAT, 50 U/mL).

### Experimental Protocols

All experimental protocols started 45 to 60 minutes after completion of the hamster cremaster preparation. Measurements of capillary dimensions were made at least 10 minutes after FITC-dextran 70 (FITC-dextran) in 0.9% saline was injected through the femoral venous cannula (0.3 mL, 20 mg/mL).

## Measurement of Anatomic and Functional Capillary Diameters

Images of randomly selected cremaster muscle capillaries were recorded on videotape during transillumination and epi-illumination (in random order). Care was taken to ensure that epi-illumination of an individual capillary was completed within a few seconds to minimize damage. As illustrated schematically in Fig 1A, the diameter of capillaries during transillumination was determined as an estimate of the anatomic capillary diameter (39 capillaries in 10 animals). The widths of RBCs (21 capillaries in 8 animals) and WBCs (12 capillaries in 7 animals) were determined from transillumination images as estimates of the functional capillary diameters available for these two types of cells. Similarly, the diameter of the FITC-dextran column in capillaries was measured during epi-illumination as an estimate of the functional capillary diameter available for flowing plasma (21 capillaries in 8 animals). In 2 animals, a fluorescent membrane dye (PKH26, Sigma) was injected with glass micropipettes into capillary networks, and anatomic capillary diameters were determined using fluorescence microscopy (18 capillaries in 2 animals).



**Figure 1.** Schematic illustration of anatomic and functional diameters of hamster cremaster muscle capillaries before (A) and after (B) exposure of a local capillary site to epifluorescence. Estimates of functional capillary diameter were obtained from measurements of the width of WBCs and RBCs and the width of the column of FITC-dextran 70. In addition, capillary tube hematocrit was determined at the epi-illuminated site relative to untreated proximal and distal capillary sites as a correlate of the ratio of functional to anatomic capillary diameter.

## Effect of RBC Velocity on Capillary Diameters

Additional experiments were performed to test for an effect of RBC velocity on diameters of RBCs and the FITC column relative to the anatomic capillary diameter. Paired measurements were made of RBC velocities and diameters of capillaries, RBCs, and the FITC-dextran column (197 capillaries in 6 animals) as described above.

## Effect of Epifluorescence on Capillary Diameters

To test for an effect of epifluorescence on capillary diameters, bright-field and fluorescence images of capillaries were obtained before and after prolonged exposure (1 to 5 minutes) of individual capillaries to epi-illumination in a subset of 7 animals (see Fig 1B). Control images of capillaries with bright-field and fluorescence microscopy were obtained as described above. After these images were obtained, individual capillaries were epi-illuminated for 1 to several minutes with a x55 objective (Leitz; NA, 0.84) until aggregation of platelets to the capillary wall became apparent. After discontinuation of the epi-illumination with the x55 objective, images were obtained again for determination of anatomic capillary diameters (10 capillaries in 7 animals), the width of the FITC-dextran column (10 capillaries in 7 animals), and the width of RBCs (6 capillaries in 4 animals).

## Effect of Epi-illumination on Capillary Tube Hematocrit

Using the x55 objective, capillary segments of  $\approx 120 \mu\text{m}$  long were observed with transillumination, and at the same time, part of each capillary segment (30 to 40  $\mu\text{m}$ ) was epi-illuminated by use of a diaphragm in the epi-illumination pathway. Images of individual capillaries were recorded until cessation of capillary RBC flow due to platelet aggregation at the epi-illuminated capillary site. Capillary tube hematocrit was determined simultaneously at  $H_{\text{local}}$  and at untreated  $H_{\text{prox}}$  and  $H_{\text{dist}}$  sites of the same capillaries (6 capillaries in 6 animals; see Fig 1<sup>+</sup>). To test whether oxygen-derived free radicals contributed to epifluorescence-induced effects on capillary hematocrit, additional measurements of capillary tube hematocrit were performed 15 minutes after starting treatment of the cremaster muscles with SOD and CAT (5 capillaries in 5 animals).

### Statistical Analysis

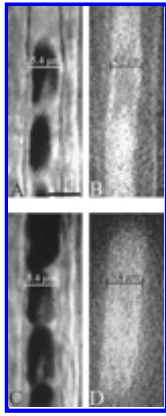
All data are expressed as mean $\pm$ SE. Paired *t* tests were used to test for differences between means.

## ► Results

### Anatomic and Functional Capillary Diameters

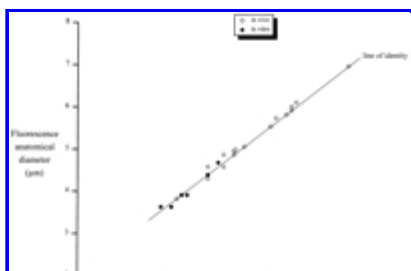
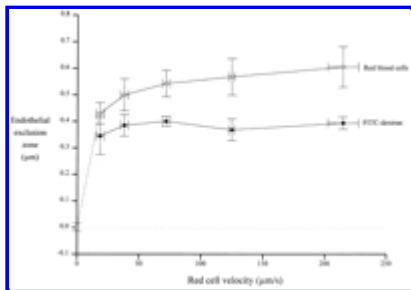
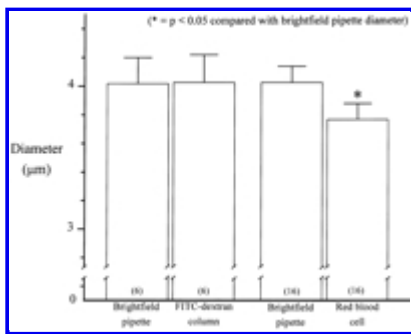
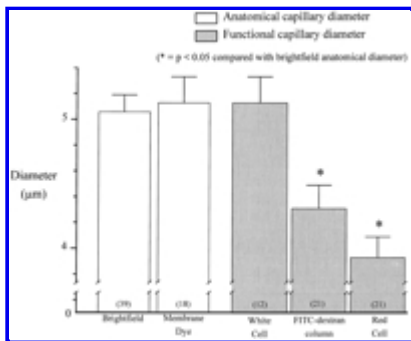
An example of a capillary visualized with bright-field and fluorescence microscopy is shown in Fig 2<sup>+</sup>. Fig 3<sup>+</sup>, top left, depicts pooled data of bright-field and fluorescent anatomic capillary diameters and average widths of WBCs, the FITC-dextran column, and RBCs. Anatomic capillary diameters (mean $\pm$ SE) averaged  $5.1\pm 0.1$  (39 capillaries in 10 animals) and  $5.1\pm 0.2 \mu\text{m}$  (18 capillaries in 2 animals) with bright-field and fluorescence microscopy, respectively. Paired measurements of the anatomic diameter with bright-field and fluorescence microscopy did not reveal a significant statistical difference. In contrast, paired estimates of anatomic (bright-field) and functional capillary diameter revealed significantly reduced functional capillary diameters available for FITC-dextran and RBCs, but not for WBCs. Functional diameters available for FITC-dextran and RBCs averaged  $4.3\pm 0.2$  and  $3.9\pm 0.2 \mu\text{m}$  (21 capillaries in 8 animals), respectively. The width of WBCs averaged  $5.1\pm 0.2 \mu\text{m}$  (12 capillaries in 7 animals), which was not different from the anatomic diameters. It must be noted that slow moving WBCs were selected for diameter measurements, because the limited amount of contrast between WBCs and the capillary wall did not allow us to make accurate measurements of fast moving WBCs. The reported WBC diameters may therefore be representative only for slow moving WBCs.

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**Figure 2.** Digitized images of a capillary segment before (A and B) and after (C and D) continuous exposure to epi-illumination for  $\approx 5$  minutes. Before epifluorescent treatment, RBC width (A) and the width of the FITC-dextran column (B) were significantly smaller than the anatomic capillary diameter (A). Treatment of the capillary with epi-illumination increased the width of RBCs (C) and the FITC-dextran column (D), without a significant effect on the anatomic capillary diameter (C). The scale bar in the lower right corner of panel A represents  $5 \mu\text{m}$ . Because of tissue movement, the lower two images are shifted upward by  $\approx 5 \mu\text{m}$  relative to the upper two images. A field diaphragm in the epi-illumination pathway was used to minimize the tissue area that was exposed to continuous epi-illumination, preventing excitation of fluorochromes in the upper and lower capillary ends in panel D.



**Figure 3.** Top left, Estimates of anatomic capillary diameters with bright-field and fluorescence microscopy after labeling of capillary endothelium with the fluorescent membrane dye PKH26 (open bars) and functional capillary diameters as determined by measuring the width of WBCs and RBCs and the width of the column of FITC-dextran 70 (shaded bars). Top right, Estimates of anatomic and functional diameters for FITC-dextran and RBCs in narrow glass tubes. Middle, Relation between RBC velocity and the thickness of apparent endothelial exclusion zones for FITC-dextran and RBCs. Bottom, All paired data values of anatomical capillary diameters determined with bright-field and fluorescence microscopy. In vivo data ( $\circ$ ) were obtained after labeling capillary endothelial cells with a fluorescent membrane dye (PKH26; see top left panel for average values). In vitro data ( $\blacksquare$ ) were obtained from narrow glass tubes filled with FITC-dextran 70 (see top right panel for average values).

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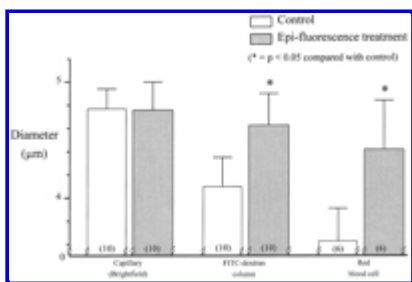
As depicted in Fig 3<sup>+</sup>, top right, the in vitro diameter of glass capillaries and the in vitro width of the FITC-dextran column were identical, and no apparent exclusion of the dextran molecules was detected in glass capillaries, which demonstrates that the apparent endothelial exclusion zone for FITC-dextran in vivo is not just a measurement artifact due to improper focus or image distortion by the SIT camera. The average in vitro width of RBCs was 0.2 to 0.3  $\mu\text{m}$  less than the in vitro anatomic and FITC-dextran diameter, which is similar to the difference of 0.2 to 0.4  $\mu\text{m}$  between the width of RBCs and the FITC-dextran column in vivo, suggesting that a fluid layer of  $\approx$ 0.1 to 0.2  $\mu\text{m}$  thick may be present between the surface of flowing RBCs and the surface accessible to FITC-dextran.

### **Effect of RBC Velocity on Capillary Diameters**

Capillaries were divided into five groups based on RBCs with velocities of  $\leq 25$   $\mu\text{m}/\text{s}$ , 25 to 50  $\mu\text{m}/\text{s}$ , 50 to 100  $\mu\text{m}/\text{s}$ , 100 to 150  $\mu\text{m}/\text{s}$ , or  $\geq 150$   $\mu\text{m}/\text{s}$ . Average RBC velocities in these groups were  $18 \pm 3$   $\mu\text{m}/\text{s}$  (SE,  $n=21$ ),  $38 \pm 3$   $\mu\text{m}/\text{s}$  ( $n=39$ ),  $72 \pm 3$   $\mu\text{m}/\text{s}$  ( $n=43$ ),  $125 \pm 2$   $\mu\text{m}/\text{s}$  ( $n=45$ ), and  $215 \pm 13$   $\mu\text{m}/\text{s}$  ( $n=49$ ). For each capillary, the thickness of the exclusion zone on the capillary wall for FITC-dextran and RBCs was calculated by subtracting the measured diameters of FITC-dextran and RBCs from paired anatomic diameters and by dividing the obtained differences by 2. It is depicted in Fig 3<sup>+</sup>, middle, that the thickness of the exclusion zone for FITC-dextran averages  $\approx 0.4$   $\mu\text{m}$ , independent of RBC velocity. For flowing RBCs, an additional fluid layer appeared to be present between the RBC surface and the FITC-dextran exclusion zone. The thickness of the RBC exclusion zone depended on RBC velocity, varying between 0.4 and 0.6  $\mu\text{m}$  at low and high RBC velocities, respectively. Additional measurements of RBC widths and anatomic capillary diameters were made in capillaries in which RBCs had momentarily stopped ( $n=14$ ). In contrast to the exclusion of flowing RBCs from the capillary wall, immobile RBCs invaded the exclusion zone for FITC-dextran, and no space could be detected between the surface of these cells and the anatomic capillary wall (Fig 3<sup>+</sup>, middle). This finding demonstrates the ability of RBCs to invade the endothelial exclusion zone for FITC-dextran when the pressure drop over capillaries is too low to maintain flow and to deform RBCs.

### **Effect of Epifluorescence on Anatomic and Functional Capillary Diameters**

Exposure of a subset of these capillaries for 1 to 5 minutes to epifluorescence significantly increased functional capillary diameters (Fig 4<sup>+</sup>) for FITC-dextran from  $4.1 \pm 0.2$  to  $4.6 \pm 0.3$   $\mu\text{m}$  and RBC width from  $3.6 \pm 0.3$  to  $4.4 \pm 0.3$   $\mu\text{m}$ , corresponding to an increase in functional capillary volume for RBCs of  $\approx 50\%$ . Epifluorescence did not affect the average anatomic capillary diameter for this subset of capillaries, being  $4.8 \pm 0.2$  and  $4.8 \pm 0.2$   $\mu\text{m}$  before and after epi-illumination.



**Figure 4.** Effect of epifluorescence on estimates of the anatomic and functional capillary diameter.

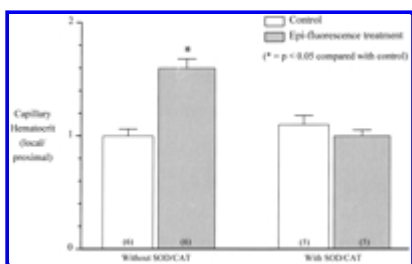
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### Effect of Epifluorescence on Capillary Tube Hematocrit

As a further test of the disparity between anatomic and functional diameter, we examined the effect of epifluorescence on the instantaneous volume of RBCs contained in the capillaries, ie, capillary tube hematocrit. If capillary functional diameter were to increase with constant RBC flux, then capillary tube hematocrit should increase in proportion to the increase in functional capillary volume. To test this, capillary tube hematocrit was simultaneously determined at  $H_{local}$  and at untreated  $H_{prox}$  and  $H_{dist}$  sites in the same capillaries. Changes in  $H_{local}$  were normalized to both  $H_{prox}$  and  $H_{dist}$  to evaluate whether epi-illumination induced local increases in capillary tube hematocrit. During epi-illumination, the ratio of  $H_{local}$  to  $H_{prox}$  increased maximally from  $1.0 \pm 0.1$  to  $1.6 \pm 0.1$  (Fig 5), and the ratio of  $H_{local}$  to  $H_{dist}$  increased maximally from  $1.1 \pm 0.0$  to  $1.6 \pm 0.1$ , corresponding to  $\approx 50\%$  to  $60\%$  increases in capillary tube hematocrit at the epi-illuminated capillary site. Treatment of the hamster cremaster muscle with SOD and CAT completely abolished the effect of epi-illumination on local capillary tube hematocrit. After treatment, the ratio of  $H_{local}$  to  $H_{prox}$  averaged  $1.1 \pm 0.1$  and  $1.0 \pm 0.1$  (Fig 5) before and during epi-illumination, respectively, and average values for the ratio of  $H_{local}$  to  $H_{dist}$  were  $1.0 \pm 0.0$  and  $1.0 \pm 0.1$ .



**Figure 5.** Effect of epifluorescence on local capillary tube hematocrit relative to untreated proximal capillary sites in the absence or presence of administered SOD and CAT.

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## Discussion

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## Summary of Findings

We have measured anatomic capillary diameters and compared these with several estimates of the functional capillary diameter defined by plasma macromolecules, RBCs, and leukocytes. The experimental data show that RBCs and fluorescent macromolecules are confined to the central core of capillaries, suggesting that inert macromolecules and RBCs have limited access to a 0.4- to 0.5- $\mu\text{m}$ -thick region on the capillary surface. Light-dye treatment increased functional capillary diameter available for dextran macromolecules and RBCs and increased the number of RBCs inside capillaries. The latter effect was completely blocked by SOD and CAT, indicating that oxygen-derived free radicals may modulate functional capillary diameter. A 0.4- to 0.5- $\mu\text{m}$ -thick endothelial surface layer that excludes macromolecules and RBCs has significant implications for capillary blood flow rheology and transcapillary exchange of macromolecules.

## Exclusion of Blood Components From a Structured Layer on the Capillary Wall

Since the early electron microscopic observations by Luft<sup>10</sup> and Shirahama and Cohen,<sup>11</sup> numerous studies have demonstrated that a surface coat is present on vascular endothelial cells. This surface coat or glycocalyx<sup>27</sup> consists primarily of carbohydrate structures that are linked to the endothelial plasma membrane by glycoproteins and glycolipids, although the exact nature and dimensions of the glycocalyx structures are unclear at this time. In 1980, Curry and Michel<sup>5</sup> hypothesized that the carbohydrate fibers of the endothelial glycocalyx form a matrix within which plasma macromolecules may have limited access. Our findings indicate that such a fibrous matrix may extend well into the lumen of mammalian capillaries and reduce functional capillary diameter for blood flow by excluding inert macromolecules and RBCs.

Several studies have revealed that certain plasma proteins bind to structures of the endothelial glycocalyx and become incorporated into the surface coat of the endothelial cells.<sup>16 28 29 30</sup> In a recent study from this laboratory,<sup>22</sup> capillary diameters available for fluorescein-labeled albumin molecules appeared to be equal or bigger than the anatomic capillary diameter. However, measurements of the width of the albumin column were not made until at least 60 minutes after systemic administration of the albumin, and incorporation of albumin in the endothelial surface layer may have resulted in an overestimation of functional capillary diameters available for free-flowing plasma macromolecules. Therefore, we chose to use fluorescein-labeled dextran macromolecules (FITC-dextran; molecular mass, 70 kD) instead of labeled plasma proteins as an inert marker of functional capillary diameter. In addition to binding of albumin to endothelial surface structures, it has been suggested that scattering of fluorescent light may have resulted in overestimates of the fluorescent capillary diameter occupied by albumin.<sup>22</sup> However, on the basis of estimates of refractive indices of plasma and tissue, scattering of light is not expected to increase the apparent fluorescent diameter relative to bright-field diameters.<sup>22</sup> Our finding that the width of the FITC-dextran column is actually 0.8  $\mu\text{m}$  smaller than the bright-field diameter further indicates that the large apparent capillary diameters for albumin might have been due to specific binding of albumin to the endothelial surface. However, if scattering of fluorescent light does indeed lead to overestimation of fluorescent diameters, the apparent exclusion of FITC-dextran from the endothelial surface might even be more pronounced than estimated from our measurements on the width of the FITC-dextran column. Measurements of capillary diameters occupied by FITC-dextran were made up to 3 hours after administration of the tracer molecules to the systemic circulation in the absence of continuous epifluorescent light exposure and compared with estimates of

the anatomic diameter of the same capillaries. Even after 3 hours, FITC-dextran still appeared to be fully excluded from the endothelial surface layer, indicating that either the endothelial glycocalyx was absolutely impermeable for these FITC-dextran molecules or that the number of FITC-dextran molecules that diffused into the endothelial surface layer was too low to be detected with fluorescence microscopy. Additional measurements (35 capillaries in one animal) were performed using dextran 70 molecules labeled with electrically neutral TR (TR-dextran, Molecular Probes) instead of negatively charged FITC to determine whether the exclusion of FITC-dextran from the endothelial surface was due to electrostatic repulsion. TR-dextran molecules were excluded from the capillary wall to a similar extent as the FITC-dextran molecules (>160 minutes; data not shown), indicating that reduced capillary diameters for dextran 70 are not due to electrostatic effects. The apparent exclusion of FITC-dextran macromolecules from the capillary wall by endothelial surface structures is in agreement with a recently proposed model of capillary exchange.<sup>5</sup> According to this model, transcapillary exchange of macromolecules is limited by a matrix of fibrous molecules that is present on the endothelial surface. This model could explain that escape of FITC-dextran molecules (molecular mass, 150 kD) from hamster cheek pouch capillaries cannot be detected with intravital fluorescence microscopy for up to 5 hours after systemic administration.<sup>31</sup> However, continuous exposure of capillaries to epifluorescence resulted in transcapillary escape of FITC-dextran within minutes.<sup>32</sup> According to the fibrous matrix model, the sudden escape of macromolecules through the capillary wall after light-dye treatment could be explained by a deleterious effect of continuous light exposure on the endothelial surface structures that form the permeability barrier. This would be consistent with our observation that light-dye treatment of capillaries allowed FITC-dextran to invade the endothelial surface layer. The similarity of these responses indicates that similar endothelial surface structures may determine both capillary permeability and the exclusion of FITC-dextran from the capillary wall and that these structures extend from the endothelial cell membrane well into the capillary lumen. However, light-dye treatment may have increased vascular leakiness by damaging the capillary wall in ways other than by destroying endothelial surface structures. Continuous light exposure of capillaries may have increased interendothelial gap width or damaged the endothelial plasma membrane. Therefore, care must be taken in comparing our observation that light-dye treatment destroys the endothelial FITC-dextran exclusion zone with reports on the effect of light-dye treatment on capillary permeability.

Additional support for exclusion of blood components from an endothelial surface layer is provided by the data on RBC dimensions. The width of RBCs was significantly less than the anatomic capillary diameter. In each capillary, however, the region between the anatomic capillary wall and the surface of RBCs appeared to be consistently thicker than the exclusion zone for FITC-dextran. Possibly, pseudopodal projections of endothelial cells may have prevented RBCs from approaching the capillary wall more closely. Alternatively, the space between RBCs and the exclusion zone for FITC-dextran may reflect a fluid layer that is required for unimpeded RBC motion in capillaries.<sup>33 34 35</sup> Both the in vitro (Fig 3<sup>+</sup>, top right) and in vivo (Fig 3<sup>+</sup>, top left and middle) data provide evidence for such a fluid layer with a thickness of  $\approx 0.1$  to  $0.2 \mu\text{m}$ . In addition, it may be possible that the luminal surface of the endothelial glycocalyx is not perfectly smooth but contains irregularities that provide limited access to FITC-dextran while excluding RBCs. In contrast to the distinct difference between anatomic capillary diameter and the width of flowing RBCs, nonflowing RBCs appeared to occupy the entire capillary cross section in a few capillaries (5 capillaries in 3 animals) during spontaneous discontinuations of blood flow (data not shown). This observation indicates that RBCs may be able to invade the endothelial exclusion zone when arteriovenous pressure drops are no longer sufficient to deform RBCs

inside capillaries.

In contrast to the exclusion of macromolecules and flowing RBCs from a 0.4- to 0.5- $\mu\text{m}$ -thick layer on the capillary wall, no difference was detected between the width of flowing WBCs and the anatomic capillary diameter. WBCs, which are bigger and stiffer than RBCs, occupied the entire anatomic capillary cross section and appear thus to be able to invade the endothelial surface layer from which macromolecules and RBCs are excluded.

### Capillary Tube Hematocrit as a Probe for Changes in Functional Capillary Volume

To test whether light-dye treatment indeed increased functional capillary diameter available for plasma macromolecules and RBCs, the effect of epifluorescence on capillary tube hematocrit was examined. Capillary tube hematocrit is proportional to the number of RBCs in capillaries and is defined as the fraction of anatomic capillary volume that is occupied by RBCs. If RBC flux remains constant, an increase in functional capillary diameter will increase capillary tube hematocrit because of reduced lengths of plasma gaps between RBCs and reduced lengths of individual RBCs. Consistent with the data on the effect of light-dye treatment on functional capillary diameter, it was found that epifluorescence increases local capillary tube hematocrit up to 60%, relative to untreated  $H_{\text{prox}}$  and  $H_{\text{dist}}$  sites (Fig 5 $\square$ ).

Before we can conclude that the observed increases in capillary tube hematocrit indeed reflect increases in functional capillary diameter, other variables that may affect capillary tube hematocrit must be examined. Alternative ways to increase capillary tube hematocrit are (1) by increasing the RBC concentration of blood flowing into the capillaries, (2) by increasing the RBC concentration inside the capillaries by leakage of water through the capillary wall, and (3) by reducing capillary RBC velocity relative to the velocity of other blood components (inverse Fahraeus effect).

In the present experiments, only a small segment of individual capillaries was exposed to epifluorescence. Light-dye treatment did not affect the velocity of RBCs flowing into or out of treated capillaries; therefore, it seems unlikely that light-dye treatment would affect the distribution of RBCs and plasma at proximal bifurcations. Nevertheless, in order to correct for spontaneous or induced changes in RBC concentrations in proximal vessels, capillary tube hematocrit was determined simultaneously at  $H_{\text{local}}$  as well as at untreated  $H_{\text{prox}}$  sites of the same capillaries. Capillary tube hematocrit increased by up to 60% at  $H_{\text{local}}$  relative to untreated capillary sites. Hence, it is reasonable to conclude that this increase in capillary tube hematocrit during local light-dye treatment is not due to an increase in RBC concentration of blood flowing into capillaries.

Theoretically, the RBC concentration of blood inside a capillary can be modulated by a change in capillary hydraulic permeability that would affect the net amount of water flowing into or out of the capillary. As illustrated in Figure 1B $\square$ , the tube hematocrit of capillary blood increased only at  $H_{\text{local}}$  and not at sites in the same capillary that were either proximal or distal to  $H_{\text{local}}$ . In order for water leakage to induce an increase in capillary tube hematocrit up to 60%, epifluorescence should increase capillary hydraulic permeability by approximately two orders of magnitude. An increase in capillary hydraulic permeability of this magnitude is unlikely, unless epifluorescence actually destroyed capillary endothelial cells, making holes in the capillary wall through which water could rapidly escape. However, such holes would also result in localized leakage of fluorescent dextran molecules from the capillary, which was not observed. A further argument against epifluorescence-induced water

leakage is that capillary tube hematocrit returned back to normal values as soon as RBCs entered the distal end of the same capillary. This observation is irreconcilable with loss of water at the epi-illuminated capillary site. Therefore, it is concluded that changes in capillary hydraulic permeability cannot explain epifluorescence-induced increases in capillary tube hematocrit.

Changes in microvascular tube hematocrit can also be caused by a change in RBC velocity relative to the velocity of whole blood.<sup>36</sup> Increases in capillary tube hematocrit due to reductions in the relative velocity of RBCs could occur if RBCs adhered to the capillary wall at the light-dye-treated capillary site. Although technical limitations did not allow us to measure adequately the flow velocity of plasma during epifluorescence, several facts argue against a relative reduction of RBC velocity as a result of adhesion to the capillary wall. First, no tendency for platelet adhesion was observed during the time that capillary tube hematocrit increased to its maximal value. In agreement with studies of Miller et al,<sup>37</sup> however, continued epifluorescence after capillary hematocrit had reached its maximum resulted in platelet adhesion. Only after platelet adhesion became apparent was it noticed that RBCs began to adhere to the capillary wall, but adhesion of RBCs to the capillary wall was never observed when capillary hematocrit increased to its maximal value. Second, consistent with the observed increase in RBC width during epifluorescence (Fig 4<sup>+</sup>), RBC length was reduced significantly during epi-illumination-induced increases in capillary tube hematocrit. In contrast, the length of adhering RBCs after continuation of epifluorescence increased significantly. Finally, although no attempts were made to determine the flow velocity of plasma, platelets were seen flowing through capillaries together with RBCs. During epifluorescence, no obvious reduction of RBC velocity relative to platelet velocity was observed. The lack of apparent RBC adhesion, the fact that the ratio of proximal to distal capillary tube hematocrit was 1 at all times, the decrease in RBC length rather than an increase, and the constant RBC velocity relative to platelet velocity make us conclude that a reduction of RBC velocity relative to whole blood velocity is not responsible for epifluorescence-induced increases in capillary tube hematocrit.

These findings support the possibility that increases in capillary tube hematocrit are due to local increases in functional capillary diameter as a result of light-dye treatment. Although the experiments with SOD and CAT indicate that oxygen-derived free radicals may be involved in this process, the exact nature of the endothelial surface structures that supposedly have been affected by light-dye treatment still remains to be identified.

### Conclusion

On the basis of these data, we conclude that a 0.4- to 0.5- $\mu\text{m}$ -thick endothelial surface layer represents the true active interface between the capillary wall and blood components, such as inert plasma macromolecules and RBCs within hamster cremaster muscle capillaries. This finding is consistent with results from a recent study in which binding of fluorescent lectins to specific carbohydrate structures indicated that the endothelial glycocalyx *in vivo* may be 0.45  $\mu\text{m}$  thick.<sup>18</sup> These estimates of the *in vivo* thickness of the capillary endothelial surface coat are considerably greater than previous estimates obtained from electron microscopic studies, which may have underestimated the true glycocalyx dimensions because of dehydration of the extracellular matrix.

## ► Selected Abbreviations and Acronyms

CAT	= catalase
FITC	= fluorescein isothiocyanate
H <sub>dist</sub>	= site distal to H <sub>local</sub>
H <sub>local</sub>	= epi-illuminated capillary site
H <sub>prox</sub>	= site proximal to H <sub>local</sub>
NA	= numerical aperture
RBC	= red blood cell
SOD	= superoxide dismutase
TR	= Texas red
WBC	= white blood cell

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