Observation of Erythrocyte Dynamics in the Retinal Capillaries and Choriocapillaris Using ICG-Loaded Erythrocyte Ghost Cells

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Purpose. To find evidence of retinal vasomotion and to examine the relationship between erythrocyte dynamics and previously observed high-frequency pulsatile blood flow through the choriocapillaris.

Methods. An osmotic shock technique was used to encapsulate indocyanine green (ICG) dye in erythrocyte ghost cells at a concentration that produced maximum cell fluorescence. By obviating the plasma staining that results from aqueous ICG's high affinity for plasma proteins, high contrast was maintained between reinjected ICG-loaded erythrocytes and their plasma background. High-speed, high-magnification ICG angiograms showing individual cell movement were recorded from the intact eyes of four monkeys and three rabbits for periods up to 30 seconds.

Results. In monkey retinal perifoveal capillaries, numerous erythrocytes were seen to pause for as long as 20 seconds and then resume transit. Similar pausing behavior was observed in the subfoveal choriocapillaris. Individual erythrocytes also were seen to pause in the rabbits' choriocapillaries below the medullary rays, where visualization of the cells was uninhibited by overlying retinal vasculature or dense pigment.

Conclusions. Reinjected ICG-loaded erythrocytes permit routine visualization of retinal capillary and choriocapillaris hemodynamics of the intact eye. It is speculated that erythrocyte-pausing in both microcirculations facilitates metabolic exchange across capillary walls. In retinal capillaries, pausing is presumed to result from vasomotion—which has been postulated as necessary for the inhibition of retinal edema—and in choriocapillaries, to result from the shifting distributions of local perfusion pressures within the network of capillary vessel segments that comprise each lobular area of the choriocapillaris vascular plexus. (*Invest Ophthalmol Vis Sci.* 2008;49:5510–5516) DOI: 10.1167/iovs.07-1504

Seldom is attention paid to the fact that blood is a shear-thinning non-Newtonian fluid; instead, blood usually is treated as if it were waterlike and not a homogeneous mixture of liquid (plasma) and solid particles (blood cells, especially erythrocytes, or red blood cells [RBCs]). Limitations inherent in conventional angiography contribute to ignoring that plasma flow does not necessarily reflect RBC dynamics, which is far more important than that of plasma to the metabolic efficiency of a particular circulation. Even in the retinal capillaries, where RBCs deform to pass through one at a time, in boxcar fashion, they cannot be visualized in conventional angiogram images. In both conventional sodium fluorescein and indocyanine green angiography (SFA and ICGA), the observed fluorescence arises from dye molecules bound primarily to blood plasma, not RBCs.[1,2] Consequently, phenomena of potential metabolic significance involving RBC movement cannot be directly observed in either the retinal or choroidal vasculatures. In that regard, there are two such phenomena that were the focus of this investigation, one involving the retinal vasculature and one the choroidal.

With regard to the retina, vasomotion is of great interest, particularly in light of a compelling hypothesis that almost all the clinical findings concerning edema in diabetic maculopathy can be explained as a result of disturbances in retinal vasomotion.[3] Vasomotion, propagating waves of alternating precapillary arteriolar contraction and dilation that result in periodic suspension of RBC movement through adjacent capillaries, has been observed in every other end-arteriolar vasculature studied so far. This phenomenon has been inferred to exist in the retinal capillaries, based on oscillations in retinal oxygen tensions (3–10 cycles/min) that correspond to brain microvascular vasomotion,[4] but vasomotion-related RBC dynamics has never been directly observed in the primate eye.

With regard to the choroid, it has been demonstrated that submacular choriocapillaris blood flow is pulsatile and that its velocity is significantly higher than that of blood flow in the arteriolar vessels that feed it.[5] Analysis of high-speed ICG angiograms indicate that blood is ejected radially through choriocapillaris lobular units at approximately 2.5 mm/s[6] and that multiple such ejections occur during a single heartbeat (Fig. 1). If the high-speed pulsatile ejection of dye-tagged plasma seen in ICGAs actually were indicative of RBC movement, then choriocapillaris blood flow would be an extremely inefficient circulation from the point of view of RBC gas exchange; RBCs would have significantly less contact time with capillary walls than those moving through end-arteriolar capillaries, such as those of the retinal vasculature.

In this study, we sought evidence of retinal vasomotion and to examine the relationship between the high-frequency pulsatile blood flow observed in the choriocapillaris and RBC

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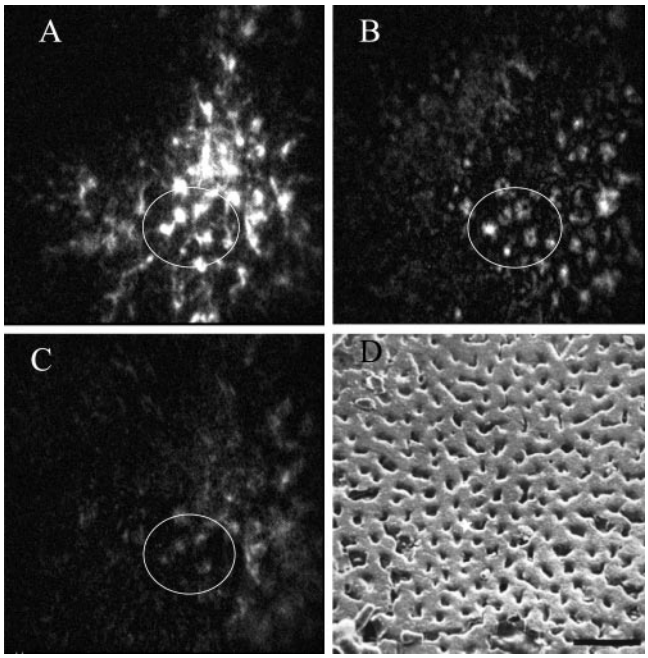

FIGURE 1. (**A–C**) High-speed (30 fps) ICG angiogram images of a Rhesus monkey eye, acquired and sequentially subtracted according to a previously described method[5] to show choriocapillaris dye filling. These images correspond to the three times at which peak filling occurred in the encircled cluster of lobular areas during a single intraocular blood pressure pulse in the eye. They were separated from each other by approximately 0.2 second. Note that the intensity of each successive pulse through the circled lobules decreases. It may be that perfusion pressure is not uniform throughout each lobular area, and that blood flow may stop temporarily through isolated sublobular areas because local perfusion pressures are too small to move blood against capillary segment resistance. Conceivably, a resonance between the intraocular and blood pressures produces the multiple ejections, each smaller than the preceding one, until the elastic tension in the arteriole is exhausted. (**D**) Corrosion cast of a portion of the subfoveal choriocapillaris area of a monkey eye. The area shown corresponds approximately to that of a large choriocapillaris lobule. Scale bar, 50 μm. The image of the cast is reprinted with permission from Shimizu K, Ujiie K. *Structure of Ocular Vessels*. Tokyo: Igaku-Shoin; 1978:75.

dynamics by direct observation of fluorescent RBCs intravenously injected into the circulation. By encapsulating ICG within RBCs, ICG concentration can be controlled so as to produce peak fluorescence, while avoiding plasma staining, thereby maintaining the high contrast needed for imaging individual cells in capillaries. Thus, ICG-loaded RBCs are ideal for studying dynamics of the particulate component of blood in the ocular circulations under normal physiological conditions.

METHODS

Loading ICG into RBCs

When in a hypotonic solution, RBCs increase in volume, causing 200- to 500-nm pores to open transiently in the RBC cell membrane.[7] During this time two-way exchange of high-molecular-weight substances can take place across the cell membrane. Thus, substances like ICG, added to the solution, may enter the RBCs. Then, raising the salt concentration of the solution to that of normal plasma closes the pores, and the cells return to an osmotically competent state, trapping the added substances inside. Subsequently, the remaining unentrapped substance can be washed away. ICG was encapsulated in human RBCs by modification of a previously reported procedure of hypotonic dialysis, isotonic resealing, and reannealing as previously reported.[8] After reinjection into the bloodstream, RBCs with encapsulated drugs appear to have a normal lifespan of up to 120 days. Use of RBCs as a drug delivery system has been extensively investigated in vitro,[9–12] and the concept was recently validated in vivo.[13–15]

Experimental Animal Angiography

High-speed ICG angiograms were acquired with a conventional fundus camera (Fundus Flash II; Carl Zeiss Meditec, Oberkochen Germany) modified by replacing its xenon flash lamp with an 805-nm wavelength diode laser light source. The diode laser was configured to deliver 1-ms pulses at a 30-Hz frequency, in synchrony with image acquisition by a charge-coupled device (CCD) video camera (Opteon; VISICS Corporation, Wellesley, MA) attached to the fundus camera's eyepiece head.

Three pigmented (Dutch-belted) rabbits, weighing between 2.61 and 2.72 kg, and 4 Rhesus monkeys, weighing between 5.8 and 8.9 kg, were subjects for the study, each being used multiple times. The rabbits were anesthetized by intramuscular injection of ketamine (50 mg/kg)/xylazine (5 mg/kg) and their eyes dilated by topical application of atropine sulfate and phenylephrine (0.5% solutions). Positioned in front of the fundus camera, eyelids of the subject's eye were retracted by insertion of a speculum, and the cornea was anesthetized by topical application of 0.5% lidocaine hydrochloride, after which a hard contact lens was placed on the cornea. An infusion catheter was then inserted prograde into an ear vein to provide a route for RBC or ICG dye injection. For monkeys, the same procedure was used, except that anesthesia consisted of intramuscular injection of ketamine HCl, followed by intubation and then halothane inhalation, and RBC and ICG dye injections were made via a saphenous vein catheter.

For angiography with injected ICG-loaded RBCs, a 0.2-mL bolus of packed cells (70% Ht) followed by a 1-mL saline flush was injected into the monkeys, and a 0.1-mL bolus of cells followed by a 0.5-mL saline flush was injected into the rabbits. For angiography with aqueous ICG dye, a 0.1-mL bolus of 25 mg/mL ICG followed by a 1-mL saline flush was injected into the monkeys, and 0.05 mL of 25 mg/mL ICG followed by a 0.5-mL saline flush was injected into the rabbits. One monkey was a subject twice, and three were subjects once. In the monkey subjects, the field of view was centered on the fovea. In the rabbits, the field of view was centered on an area temporal to the optic disc and inferior to the medullary rays. One rabbit was a subject twice, one three times, and one four times. None of the animals exhibited an adverse reaction to repeated RBC or aqueous ICG dye injections. The experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

Dilution of Injected ICG-Loaded RBCs by Circulating Cells

Assuming blood accounts for 7% of the body mass,[16] the 5.8-kg monkey would have had a total blood volume of approximately 0.4 L, of which 180 mL would be RBCs. Therefore, the injected 0.2 mL bolus of packed (70% Ht) ICG-loaded RBCs— equivalent to 0.14 mL of RBCs—amounted to only 0.07% of the monkey's total blood volume. Nevertheless, during initial transit through the ocular vasculatures, the density of ICG-loaded RBCs was so high that it was difficult to identify individual cells, especially due to the high density of ICG-loaded cells in the choroid; the choriocapillaris appeared to scintillate. However, at 9 minutes after injection, sufficient dilution by circulating nonfluorescent cells had taken place that individual fluorescent RBCs could be distinguished in both the retinal and choroidal circulations. The cells in the retinal circulation appeared as distinct points of bright fluorescence, whereas those in the choroidal circulation appeared less bright and fuzzy, since they lay beneath the layer of pigmented retinal epithelium which scattered the fluorescence arising from each RBC. Because of further dilu-

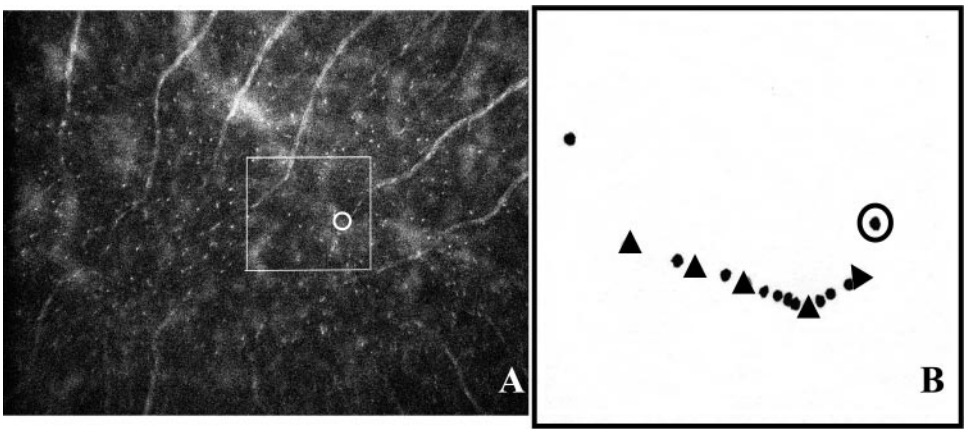

FIGURE 2. (**A**) An enhanced image from a 9-minute post–RBC-injection angiogram sequence. Note the numerous ICG-loaded RBCs that are visible. The *boxed area* includes a quadrant of the perifoveal capillary region and defines the fundus area represented in (**B**). The *circle* in (**A**) corresponds to the *circle* in (**B**) and to the six *circles* in Figure 3. Assuming the foveal avascular zone (FAZ) to be approximately 500 μm in diameter, then the *boxed area* has about a 1-mm diagonal. (**B**) Plot of the movements of two RBCs tracked in Figure 3, thereby defining the shape of the perifoveal capillary and demonstrating that the two tracked RBCs (one represented by *dots*, and the other by *triangles*) moved through the same capillary.

tion by circulating nonfluorescent cells, generally approximately 20 minutes after injection, only a few ICG-loaded RBCs were visible.

Tracking Individual RBCs in the Monkey Retinal Circulation

During the 10- to 20-second-long angiographic sequences recorded after ICG-labeled RBC injection, numerous cells in the retinal vasculature were seen to pause for a few seconds and to then resume movement; others were seen to traverse the field of view without pausing. When viewing the angiogram sequences at video speed (25 images/sec) or when playing subsets of image sequences in looplike fashion, it was relatively easy to track individual fluorescent cells. (To demonstrate this, the two original video sequences from which the individual frames for Figures 3, 4, and 5 were extracted are provided. See Movies S1 and S2 online at http://www.iovs.org/cgi/content/full/49/12/5510/DC1.) Despite the relatively high contrast between fluorescent RBCs and their background, it is much more difficult to track individual cells from frame to frame in sequences of still images. Nevertheless, an attempt is made to demonstrate RBC motion through the retinal perifoveal capillaries in Figures 2 and 3. Figure 2A is an image from a high-speed angiogram, centered approximately on the fovea of one of the Rhesus monkey eyes, which has been enhanced to show the retinal vascular arcades for orientation. Note that numerous ICG-loaded RBCs are visible. The boxed area indicates the quadrant of the perifoveal capillary region from which the individual images in Figure 3 were taken; the circled RBC corresponds to the same reference RBC circled in Figure 2B and six of the images in Figure 3. Assuming the foveal avascular zone (FAZ) to be approximately 500 μm in diameter, then the boxed area has about a 1-mm diagonal. The drawing to the right (Fig. 2B) also corresponds to the boxed area; it summarizes the movements of two RBCs tracked in Figure 3, thereby defining the shape of the perifoveal capillary and demonstrating that the two RBCs were moving through the same capillary.

The two columns to the left in Figure 3 track progression of the first RBC from Figure 2B through a retinal capillary immediately adjacent to the FAZ, as indicated by the arrows. The right-hand column tracks the second RBC through the same capillary. For orientation, one of several RBCs that remained stationary throughout all the images in the figure is circled at the beginning and end of each column. Time elapsed from the first image in which the tracked RBC entered the field of view (i.e., 0.00 second) is indicated beside each image. The first RBC moved steadily for 0.36 seconds and then paused for 1.25 seconds, made a very small movement during the next 0.20 second, and then continued to remain in place for an additional 0.68 second. During the next 0.16, it moved farther toward the edge of the FOV and then disappeared very quickly.

A second RBC entered the same capillary approximately 0.06 second later and is tracked in the right-hand column, starting from a point between where the first one was at 0.00 second and at 0.12 second to exactly the same point where the first RBC was at 2.92 seconds Whereas, it took the first RBC 0.52 second (not including the 2.40 seconds it paused) to move from its 0.12-second position to its 2.92-second position (0.56 mm, using the diameter of the FAZ as a scale), the second RBC traversed 0.38 mm through the same capillary in 0.48 second. Thus, the average velocity of the first RBC was 1.08 mm/s, whereas that of the second RBC was 0.79 mm/s. One other RBC in a different capillary bordering the FAZ was tracked at an average velocity of 0.93 mm/s, during which time it paused for 0.60 second. The behavior of these RBCs was typical of those observed throughout the angiogram FOVs of the monkey eyes observed, with duration of pauses ranging from approximately 2 to 15 seconds.

Tracking Individual RBCs in the Monkey Choroidal Circulation

Figure 4 shows an angiogram image from the sequence acquired 9 minutes after RBC injection. The boxed area is centered on the FAZ, from which the images shown on the right were acquired. The arrow indicates an RBC in the choriocapillaris. In the image sequence, that cell gradually became visible during the 0.16 seconds it took to rise to the plane of the choriocapillaris. Once there, it paused from 0.12 second until 0.72 second. Then, it quickly moved slightly laterally and down. Note that at 0.76 and 0.80 seconds it becomes less bright, and its diameter becomes larger due to increased light scatter as the cell moves deeper into the choroid; at 0.84 second, it was no longer visible.

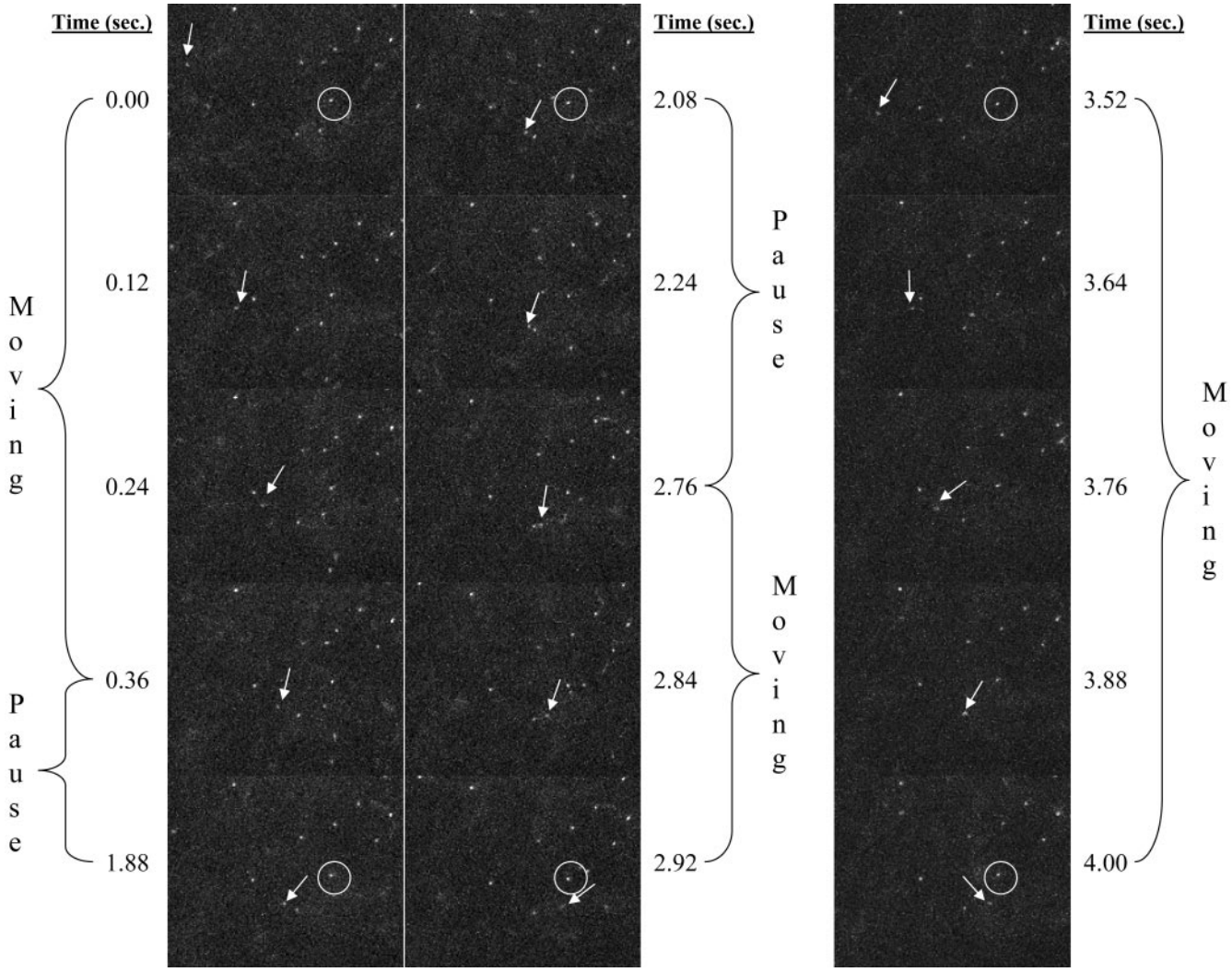

FIGURE 3. The left-hand two columns track progression of one RBC through a retinal capillary immediately adjacent to the FAZ (*arrows*). The right-hand column tracks a second RBC through the same capillary (*arrows*). For orientation, one of several RBCs that remained stationary throughout all the images in the figure is circled at the beginning and end of each column. Because only selected images from the 100 that comprised the angiogram sequence (recorded at 25 images/s) could be shown, the time elapsed from the first image in which the tracked RBC entered the field of view is indicated beside each image.

Tracking Individual RBCs in the Rabbit Choroidal Circulation

Better visualization of choriocapillaris blood flow was possible in the rabbit eyes, since below the medullary rays, there is neither overlying retinal capillary network nor dense RPE to obscure it. As with the monkey angiograms, an attempt was made to demonstrate the motion of the RBCs seen in a movie sequence. Selected images from an angiogram sequence are shown in Figure 5, and the motion of one of the RBCs is tracked. As in the monkey eye in Figure 4, as the RBC rose to the choriocapillaris plane from 0.00 to 0.12 second, it became brighter and more distinct as its diameter lessened. This RBC paused for nearly 6 seconds, at which time it began to shift laterally from its paused position (indicated by the short arrows at the top and bottom images of the right-hand column), and then it abruptly disappeared. Not including pause times, several of these cells having lateral motion through the choriocapillaris were calculated to have velocities of approximately 2.0 mm/s; this is slower than the approximate 2.5 mm/s velocity for ICG-stained blood plasma transiting the choriocapillaris lobules of the rhesus monkey reported previously.[6]

DISCUSSION

The ability to visualize capillaries in the ocular fundus depends on having both sufficient spatial resolution and sufficient contrast between the capillaries and their background. Diffraction-limited spatial resolution of a normal eye is sufficient for visualizing the retinal perifoveal capillaries using reflected light, but contrast between the capillaries and their background is insufficient. Visualization of the choriocapillaris is more problematic because of the overlying macular xanthophyll and RPE. Even when reflected near-infrared (IR) light, which is transmitted by xanthophyll and RPE, is used, the pigment granules scatter the light, thereby reducing spatial resolution to the extent that adjacent capillaries cannot be distinguished from each other. Moreover, as with the retinal capillaries, there is insufficient contrast between choriocapillaries and their background.

Poor contrast can be overcome by filling capillaries with fluorescent dyes. Fluorescein fluorescence results in high contrast between the retinal perifoveal capillaries and the dark background of the underlying macular xanthophyll, and since

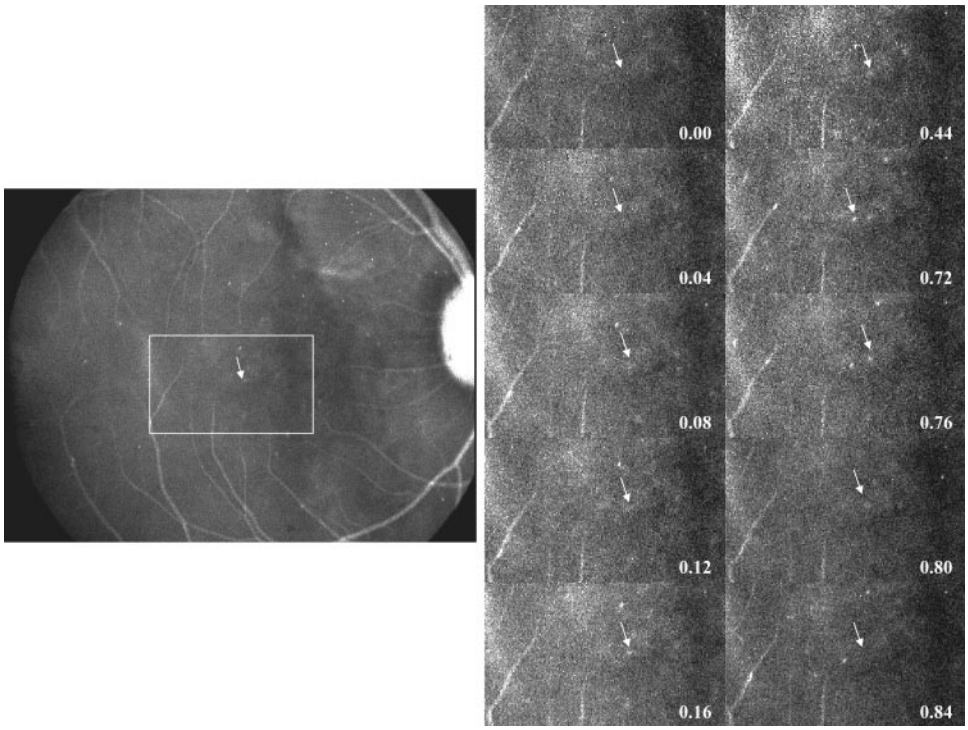

FIGURE 4. Images from a monkey angiogram sequence acquired 9 minutes after RBC injection. The *boxed area* indicates an area centered on the FAZ, from which the images shown on the right were acquired. Time elapsed from the first image in which the tracked RBC entered the field of view is indicated in the corner of each image. The *arrows* track one RBC in its transit through the choriocapillaris. Note that the RBC became less bright, and its diameter became larger because of the increased light scatter as the cell moved deeper into the choroid. At 0.84 second, it was no longer visible.

the spaces between adjacent capillaries exceed the spatial limitation of approximately 7 μm imposed by the optics of the eye, the capillary net can be visualized. Likewise, near-IR fluorescence from ICG dye produces contrast between the choriocapillaries and their background, but as the light is scattered by pigment granules, spatial resolution is reduced to approximately 11 μm, and since the spaces between adjacent choriocapillaris segments are smaller than that, one segment cannot be distinguished from another. Consequently, the smallest choriocapillaris plexus vascular components that can be visualized in conventional angiograms are the 200- to 400-μm diameter so-called lobular areas demonstrated in the Figure 1. As indicated in Figure 1D, the lobular areas consist of many short, interconnected capillary segments whose 20- to 30-μm diameters usually are greater than the approximately 10-μm spaces between them.

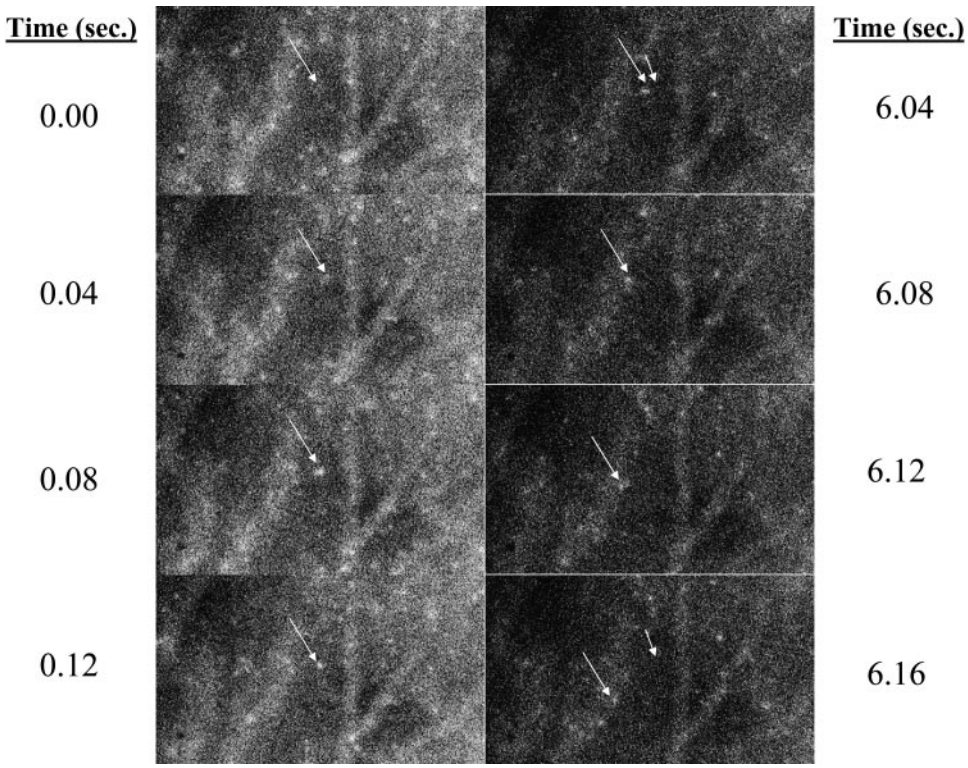

FIGURE 5. Images from a rabbit angiogram, with *arrows* tracking the position of an ICG-loaded RBC in the choriocapillaris. This particular RBC paused for nearly 6 seconds, at which time it began to shift laterally from its paused position (indicated by the *short arrows* in the *top* and *bottom* images of the right-hand column), and then it abruptly disappeared.

Table 1. Comparison of RBCs from Different Species

Species	Diameter (μm)	Mean Volume (μm^3)
Homo sapiens	7.3	88
Macaca mulatta	7.2	78
Oryctolagus cuniculas	6.1	71

Once filled with dye-stained plasma, a blood vessel exhibits steady state fluorescence, inhibiting visualization of any blood-borne particles, even if the particles themselves were fluorescent. However, it is possible to visualize RBCs in capillaries if they, rather than the plasma, emit fluorescence and if separation between adjacent RBCs exceeds the diffraction limited resolution of the optics of the eye (~7 μm for the perifoveal capillaries and 11 μm for the choriocapillaris[17]). These conditions were met by entrapping ICG dye in RBCs and reinjecting a small bolus of them; during transit to the ocular vasculatures, they are diluted in circulating blood to the extent that individual ICG-loaded RBCs were separated by more than 11 μm, making visualization of them possible.

Use of Human RBCs

Ideally, these studies would have been conducted using autologous reinjection of each subject's RBCs, but that was not a realistic option. In the case of the monkeys, it was not feasible to obtain blood for processing before performing angiography, and rabbit RBCs are not amenable to dialysis encapsulation, yielding only 8% to 15% encapsulation, and the cells often gel during the annealing phase of encapsulation.[18] Therefore, human RBCs, all from the same donor, were used instead. Human, monkey and rabbit RBCs all have the same biconcave disc shape, and as shown in Table 1, they all have nearly the same dimensions and volumes. Furthermore, no immune or allergic responses have been observed, even after multiple RBC injections made on the same or different days. Of course, over time the injected cells do undergo eryptosis, but not all at once. Therefore, the tiny amounts of foreign hemoglobin that are gradually released did not seem to adversely affect the subject animals. More important, even if the foreign cells were to evoke some immune or allergic response, it would be unlikely that the time course of the response would manifest itself in such a way as to affect the hemodynamic observations made within 20 minutes of cell injection. When aqueous dye angiograms were performed at the end of experiments, no abnormal filling was observed.

RBC Dynamics in the Retinal Perifoveal Capillaries

Other investigators have used various methods for measuring perifoveal capillary blood flow velocity, as summarized recently by Funatsu et al.,[19] including their own of scanning laser ophthalmoscopic fluorescein angiography. The summarized velocities ranged from 1.51 to 3.28 mm/s in normal human eyes and from 1.00 to 2.89 mm/s in diabetic eyes. Mention also was made of one report of 1.2 mm/s in a normal anesthetized cynomolgus monkey eye. (Our individual RBC velocity measurements of 0.73, 0.93, and 1.08 mm/s in the peripheral FAZ capillaries of a Rhesus monkey only underscore that measurements of discrete dynamic events in any biological system are variable.) Funatsu et al. noted that all the earlier reports presented only mean blood velocity data and that there was no mention of the velocity variations in single capillaries they themselves observed in both normal eyes and those with macular edema. However, they were no more certain than the earlier investigators as to the exact nature of the "fluorescent dots" whose velocities were being measured; speculation ranged from aggregations of platelets, to leukocytes, to fluorescent plasma between "erythrocyte rouleaux formations"—because "erythrocytes do not stain with fluorescein."[19] Nor were they certain as to why the velocity variations occur, but they did speculate that, among other causes, the variations in velocities of different dots moving through the same capillary may be due to the pulsation of blood flow. We suggest that indeed "pulsations in blood flow" due to vasomotion is the most likely explanation.

Although intraocular pressure (IOP) was measured and determined to be within normal range before angiographic observations and blood pressure monitored throughout the monkey experiments, it was not technically feasible to measure IOP pressure during angiogram acquisition, and so no correlation was made between ocular perfusion pressure and the motion of RBCs. However, since the effects of perfusion pressure fluctuations are asserted globally across the retinal vasculature, they would not likely account for the out-of-phase distribution of RBC pausing observed to occur in each subject eye. This out-of-phase distribution cannot be appreciated in the still images of the figures presented, but it is quite apparent in the video sequences from which they were taken.

The high-speed angiograms made of Rhesus monkey eyes demonstrated RBC dynamics consistent with vasomotion, manifest as alternating perfusion of adjacent capillaries at frequencies in the range from 1 to 20 per minute. Vasomotion is seen in most vascular beds, and its existence in the retinal circulation has been postulated. Bek[3] suggested that retinal vasomotion has been scarcely studied because "no methods have been used that are suitable for studying dynamic phenomena taking place between approximately five seconds and five minutes." To our knowledge, the angiograms made during this study are the first direct visualizations made of this phenomenon in the intact primate eye. It was further postulated that vasomotion is an important mechanism for homogenous oxygen distribution in retinal tissue and that disturbances in vasomotion may underlie diabetic maculopathy.[3] That being the case, eventually high-speed angiography using autologous reinjection of ICG-loaded RBC ghost cells would be a sensitive diagnostic tool for early detection of diabetic maculopathy and for monitoring its progression and response to treatment.

Efficiency of RBC Metabolic Exchange in the Choriocapillaris

The approximate 2.0 mm/s speed of RBCs in the rabbit choriocapillaris—apparently during intraocular blood pressure systole—as determined by the present study, is slower than, but consistent with, the 2.5 mm/s velocity previously determined from aqueous ICG angiogram data from the monkey.[5] Also it is in the range of the 2.45 $\pm$ 0.48 mm/s average velocity of fluorescent ICG-tagged leukocytes in the monkey choroid, as determined by Takasu et al.,[20] but it is about double the 1.2 mm/s velocity through the monkey retinal capillaries reported by Tanaka et al.[21] It is interesting that Takasu et al. also commented that the ICG-tagged cells seen in the sub-FAZ choroid were "dim fluorescent dots" compared with the "distinct fluorescent dots seen moving in the fundus," the same difference we noted in our monkey angiograms. However, in pigmented rabbit choroid, they measured only a mean leukocyte speed of 0.48 $\pm$ 0.14 mm/s, compared with the maximum of 2.0 mm/s seen in this study using fluorescent RBCs.

Perhaps the most interesting observation was that RBC movement through the choriocapillaris appeared analogous to that observed in the retinal capillaries; that is, numerous ICG-loaded RBCs moved very slowly and/or paused during choriocapillaris transit, but the mechanism that causes such behavior is not clear. Nevertheless, because the diameters of the cho-

riocapillaries are so large that several RBCs can move through them abreast, it is assumed that for every such paused cell observed, there are many more adjacent, unseen non-ICG-loaded RBCs that do the same. As indicated earlier, to visualize individual fluorescent cells (as in the images of Fig. 5 and the video sequence from which they came), they must be separated from each other by more than 11 μm, and presumably the intervening space would be occupied by nonfluorescent cells. Intuitively, such behavior by many RBCs would enhance the efficiency of RBC metabolic exchange, compared with that which would be expected if all RBCs moved through choriocapillaris vessels in the high-speed bursts indicated by conventional high-speed ICGA, permitting only very short contact times with capillary walls. However, the mechanism of pausing is a matter of conjecture at this time.

The so-called lobular areas seen in angiograms result from blood flow patterns determined by the network of perfusion pressure gradients extant among the interspersed arterioles and venules connected to the choriocapillaris plexus. It has been shown that filling phase relationships between adjacent lobuli, and even numbers and sizes of lobuli, change over time, presumably due to chances in the relative pressures within the arterioles.[6] These lobules are the smallest functional choriocapillaris unit that can be observed in conventional angiograms, but, as shown in Figure 1D, each lobule consists of hundreds of short capillary segments. Each such area is fed from a centrally located arteriole, accounting for the radial lobular filling observed in high-speed angiograms. Dye movement through the individual choriocapillaris segments has never been observed, because they are beyond the spatial resolution of fundus cameras, so it is unknown whether blood moves through all the capillary segments of a given lobular area during each ejection of blood from its feeding arteriole during each heartbeat. It is possible, however, to visualize single ICG-loaded erythrocytes within various— but probably not adjacent—such segments. We speculate that perfusion pressure is not uniform throughout each lobular area, and when RBCs are observed to pause, it is during periods where blood flow stops temporarily through isolated sublobular areas because local perfusion pressures are too small to move blood against capillary segment resistance.

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References

1. Wessing A. *Fluorescein Angiography of the Retina.* St. Louis: C.V. Mosby; 1969:13.
2. Ott P. Hepatic elimination of indocyanine green with special reference to distribution kinetics and the influence of plasma protein binding. *Pharmacol Toxicol.* 1998;83(suppl 5):28–30.
3. Bek T. Diabetic maculopathy caused by disturbances in retinal vasomotion: a new hypothesis. *Acta Ophthalmol Scand.* 1999;77:376–380.
4. Braun RD, Linsenmeier RA, Yancey CM. Spontaneous fluctuations in oxygen tension in the cat retina. *Microvascular Res.* 1992;44:73–84.
5. Flower RW. Extraction of choriocapillaris hemodynamic data from ICG fluorescence angiograms. *Invest Ophthalmol Vis Sci.* 1993;34:2720–2729.
6. Flower RW. Variability in choriocapillaris blood flow distribution. *Invest Ophthalmol Vis Sci.* 1995;36:1247–1258.
7. Baker RF, Gillis NR. Osmotic hemolysis of chemically modified red blood cells. *Blood.* 1969;33:170–178.
8. Magnani M, Rossi L, Brandi G, Schiavano GF, Montroni M, Piedimonte G. Targeting antiretroviral nucleoside analogues in phosphorylated form to macrophages: in vitro and in vivo studies. *Proc Natl Acad Sci U S A.* 1992;89:6477–6481.
9. DeLoach R. Encapsulation of exogenous agents in erythrocytes and the circulating survival of carrier erythrocytes. *J Appl Biochem.* 1983;5:149–157.
10. DeLoach JR, Sprandel U, eds. *Red Blood Cells as Carriers for Drugs*. Basel, Switzerland; 1986.
11. Ropars C, Chassaigne M, Nicolau C. *Red Blood Cells as Carriers for Drugs*: *Potential Therapeutic Applications*. Oxford, UK: Pergamon Press; 1987.
12. Rossi L, Brandi G, Schiavano GF. Heterodimer-loaded RBCs as bioreactors for slow delivery of the antiviral drug azidothymidine and the antimycobacterial drug ethambutol. *AIDS Res Hum Retrovir.* 1999;15:345–353.
13. Rossi L, Serafini S, Cenerini L, et al. RBC-mediated delivery of dexamethasone in patients with chronic obstructive pulmonary disease. *Biotechnol Appl Biochem.* 2001;33:85–89.
14. Rossi L, Castro M, D'Orio F, et al. Low doses of dexamethasone constantly delivered by autologous RBCs slow the progression of lung disease in cystic fibrosis patients. *Blood Cells Mol Dis.* 2004;33:57–63.
15. Annese V, Latiano A, Rossi L, et al. RBCs-mediated delivery of dexamethasone in steroid-dependent IBD patients-a pilot uncontrolled study. *Am J Gastroenterol.* 2005;100:1370–1375.
16. Cameron JR, Skofronick JG, Grant RM. *Physics of the Body*. 2nd ed. Madison, WI: Medical Physics Publishing; 1999:182.
17. Flower RW. Optimizing treatment of choroidal neovascularization feeder vessels associated with age-related macular degeneration. *Am J Ophthalmol.* 2002;134:228–239.
18. DeLoach JR. Hypotonic dialysis encapsulation in RBCs of mammalian species. *Bibl Haematol.* 1985;51:1–6.
19. Funatsu H, Sakata K, Harino S, Okuzawa Y, Noma H, Hori S. Tracing method in the assessment of retinal capillary blood flow velocity by fluorescein angiography with scanning laser ophthalmoscope. *Jpn J Ophthalmol.* 2006;50:25–32.
20. Takasu I, Shiraga F, Okanouchi T, Tsuchida Y, Ohtsuki H. Evaluation of leukocyte dynamics in choroidal circulation with indocyanine green-stained leukocytes. *Invest Ophthalmol Vis Sci.* 2000;41:2844–2848.
21. Tanaka T, Muraoka K, Shimizu K. Fluorescein fundus angiography with scanning laser ophthalmoscope. *Ophthalmology.* 1991;98:1824–1829.