Quantification of Blood-Brain Barrier Solute Permeability and Brain Transport by Multiphoton Microscopy

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Development of an optimal systemic drug delivery strategy to the brain will require non-invasive or minimally invasive methods to quantify the permeability of the cerebral microvessel wall or blood-brain barrier (BBB) to various therapeutic agents and to measure their transport in the brain tissue. To address this problem, we used laser-scanning multiphoton microscopy to determine BBB permeability to solutes (P) and effective solute diffusion coefficients ($D_{eff}$) in rat brain tissue 100–250 μm below the pia mater. The cerebral microcirculation was observed through a section of frontoparietal bone thinned with a microgrinder. Sodium fluorescein, fluorescein isothiocyanate (FITC)-dextran, or Alexa Fluor 488-immunoglobulin G (IgG) in 1% bovine serum albumin (BSA) mammalian Ringer’s solution was injected into the cerebral circulation via the ipsilateral carotid artery by a syringe pump at a constant rate of ~3 μl/min. P and $D_{eff}$ were determined from the rate of tissue solute accumulation and the radial concentration gradient around individual microvessels in the brain tissue. The mean apparent permeability P values for sodium fluorescein (molecular weight (MW) 376 Da), dextran-4k, -20k, -40k, -70k, and IgG (MW ~160 kDa) were 14.6, 6.2, 1.8, 1.4, 1.3, and 0.54 × 10⁻⁶ cm/s, respectively. These P values were not significantly different from those of rat pial microvessels for the same-sized solutes (Yuan et al., 2009, “Non-Invasive Measurement of Solute Permeability in Cerebral Microvessels of the Rat,” Microvasc. Res., 77(2), pp. 166–73), except for the small solute sodium fluorescein, suggesting that pial microvessels can be a good model for studying BBB transport of relatively large solutes. The mean $D_{eff}$ values were 33.2, 4.4, 1.3, 0.89, 0.59, and 0.47 × 10⁻⁶ cm²/s, respectively, for sodium fluorescein, dextran-4k, -20k, -40k, -70k, and IgG. The corresponding mean ratio of $D_{eff}$ to the free diffusion coefficient $D_{free}$, $D_{eff}/D_{free}$, were 0.46, 0.19, 0.12, 0.12, 0.11, and 0.11 for these solutes. While there is a significant difference in $D_{eff}/D_{free}$ between small (e.g., sodium fluorescein) and larger solutes, there is no significant difference in $D_{eff}/D_{free}$ between solutes with molecular weights from 20,000 to 160,000 Da, suggesting that the relative resistance of the brain tissue to macromolecular solutes is similar over a wide size range. The quantitative transport parameters measured from this study can be used to develop better strategies for brain drug delivery. [DOI: 10.1115/1.4025892]

Keywords: cerebral microvessel, brain parenchyma, minimally invasive, rat, laser-scanning, sodium fluorescein, dextran, IgG

Introduction

The blood-brain barrier (BBB) is a dynamic interface between the blood circulation and the central nervous system (CNS). While it serves as a selective barrier to water and solutes to prevent the blood-borne toxins from entering into the brain tissue, it hinders the delivery to the CNS of drugs used to treat brain diseases. To search for the strategies to improve drug delivery efficacy by modulating paracellular or transcellular pathways across the BBB, it is necessary to develop non- or minimally invasive methods to quantify BBB permeability to various therapeutic agents. Previous methods for estimating BBB permeability include intracerebral microdialysis [1], magnetic resonance imaging [2], pharmacokinetic methods [3–5], the indicator diffusion method [6], occluding single microvessel measurement [7], positron emission tomography [8], and intravital fluorescence microscopy [9,10]. Unfortunately, these methods either did not measure the driving force (solute concentration difference across the BBB) or the solute flux needed to determine the true BBB permeability or only measured the permeability P of pial microvessels at the brain surface due to the penetration limitation of the single-photon fluorescence microscopy.

Interstitium, including brain tissue, contains a matrix of fibrous molecules that creates considerable resistance to water and solutes. To quantify the transport of a therapeutic agent in brain tissue and to predict its spatial and temporal tissue concentration distributions, it is essential to determine its effective tissue diffusion coefficient $D_{eff}$. Previously, $D_{eff}$ of solutes, e.g., albumin, dextran, and IgG, have been measured in a variety of nonbrain tissues [11–16] and in brain tissues [17–23] by various techniques, e.g., fluorescence recovery after photobleaching, microdialysis; systemic and single-vessel injection of fluorescently labeled solutes; analysis of tissue-spreading images, real-time iontophoresis, or real-time pressure ejection; integrative optical imaging analysis by laser-scanning confocal or multiphoton microscopy; and magnetic resonance. In these methods, $D_{eff}$ was determined from the collected 2D images that reflected an averaged tracer distribution over a depth of the tissue sample and sometimes included the tissue surface and the superfusate layer. Possible errors in these

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measurements could occur from unmeasured changes in the solute concentration in the tissue due to averaging the distribution over a nonuniform tissue, as well as from loss of tracers through the tissue surface, resulting in an overestimation in $D_{eff}$.

Previously, a 3D in vivo method [24–26] was developed for the determination of $P$ and $D_{eff}$ in thin and translucent frog mesenteric tissue by using laser-scanning confocal microscopy. Recently, a noninvasive method was developed [27] to measure $P$ of pial microvessels at the rat brain surface. On the basis of these prior studies, the objective of the current study was to utilize the greater tissue-penetrating capability of multiphoton microscopy to simultaneously determine the permeability of the cerebral microvessel to solutes $P$ and effective solute diffusion coefficient $D_{eff}$ in the brain parenchyma. This new method also overcomes the overestimation and uncertainties in $D_{eff}$ inherent in previous methods.

Materials and Methods

Animal Preparation. In vivo experiments were conducted on adult female Sprague–Dawley rats (250–300 g, age 3–4 months) purchased from the Hilltop Laboratory Animals (Scottsdale, PA). All procedures and the animal use were approved by the Institutional Animal Care and Use Committee of the City College of New York. Rats were anesthetized with pentobarbital sodium given subcutaneously. The initial dosage was 65 mg/kg. A heating pad was used to keep the rats warm. The depth of anesthesia was monitored for the absence of withdrawal reflex to toe or ear pinch and absence of blink reflex. Usually, these criteria were satisfied in 1–1.5 h. Anesthesia was further checked every 15 min during the experiment; an additional 3 mg/dose pentobarbital was given when needed. At the end of experiments, an overdose of pentobarbital (>100 mg/kg) was administered intravenously to euthanize the animal.

The skull in the region of interest was exposed by shaving off the hair and cutting away the skin and connective tissue. A section of the frontoparietal bone (left or right) was carefully ground with a high-speed microgrinder (0–50,000 rpm, DLT 50KBU, Brasseler USA, GA) until a part of it (~4 mm × 6 mm) became soft and translucent. During the process, artificial cerebrospinal fluid (ACSF) at room temperature was applied to the surface of the skull to dispel the heat due to grinding. After grinding, the left or right carotid artery was cannulated with PE50 tubing. The rat was then placed on the stereotaxic instrument (David Kopf Instruments), and its head was fixed with two ear bars and a mouth clamp. The cerebral microvessels were observed under the objective lens of a multiphoton microscope through the thinned part of the skull. The fluorescently labeled solutes in 1% BSA Ringer solution containing 10 mg/ml BSA were then introduced into the cerebral circulation via the ipsilateral carotid artery. We selected a brain region containing postcapillary venules of 20–40 μm in diameter [30] and then collected images for a region of interest (ROI) with a volume of ~200 μm × 8 μm × 100 μm ($x$, $y$, $z$) (Fig. 1) at a rate of ~5 s per image. The corresponding pixel sizes were ~428 μm × 17 μm × 100 pixel, resulting in a spatial resolution of ~0.47 μm × 0.47 μm × 1 μm in $x$, $y$, and $z$ directions. The collected images of the ROI were then transferred to an image acquisition and analysis workstation for determining the $P$ and $D_{eff}$.

Multiphoton Microscope and Image Collection. Twelve-bit images were collected in vivo by an Ultima Multiphoton Microscopy system (Prairie Tech., Inc., WI). The vessels were observed with a 40× lens (NA = 0.8, water immersion, Olympus). For FITC and Alexa Fluor 488–labeled solutes, the excitation wavelength of the multiphoton microscope was set to 800–850 nm to observe the cerebral microvessels ~100–250 μm below the pia mater. The fluorescence solution was introduced into the cerebral circulation by a syringe pump at a constant rate of ~3 μl/min, the normal blood flow rate through the carotid artery [36,37]. It took ~10–15 s for the dye from the cannulation site at the carotid artery to reach the cerebral microvessels. The images were taken simultaneously while the fluorescence solution was introduced into the cerebral circulation via the ipsilateral carotid artery. We selected a brain region containing postcapillary venules of 20–40 μm diameter [30] and then collected images for a region of interest (ROI) with a volume of ~200 μm × 8 μm × 100 μm ($x$, $y$, $z$) (Fig. 1) at a rate of ~5 s per image. The corresponding pixel sizes were ~428 μm × 17 μm × 100 pixel, resulting in a spatial resolution of ~0.47 μm × 0.47 μm × 1 μm in $x$, $y$, and $z$ directions. The collected images of the ROI were then transferred to an image acquisition and analysis workstation for determining the $P$ and $D_{eff}$.

Image Analysis. The images were analyzed using Image J (National Institutes of Health). The collected images were reconstructed into a segment of 200 μm × 100 μm cross-sectional area ($x$–$y$) with 8 μm thickness. The temporal and spatial solute intensity (concentration) profiles $I$ ($x$, $y$, $z$) surrounding a microvessel in this cross-sectional area with 8 μm thickness of the brain tissue were determined by the ImageJ program.

Calibration. The primary assumption in calculating $P$ and $D_{eff}$ with the use of fluorescent solutes is that the fluorescence intensity $I$ is a linear function of the number of solute molecules (concentration) in the measuring field. To test the linear range of intensity versus concentration, we used the same instrument settings in the in vitro calibration experiments as those used in in vivo measurements. In vitro calibrations were described in Ref. [27]. Briefly, it was performed using a chamber with the depth of 340 μm created with the glass cover slips. Solutions of fluorescence probes were applied to fill the chamber by capillarity. The fluorescence intensity was measured in a volume of 200 μm × 8 μm × 100 μm ($x$, $y$, $z$), which began at the bottom cover slip and extended 100 μm deep into the solution. A fluorescence image was collected for each concentration. Figure 2 shows that the relationship between...
the concentration and the fluorescence intensity was linear from 0.05 to 0.2 mg/ml for sodium fluorescein (Fig. 2(a)), from 0.25 to 1.25 mg/ml for FITC-dextran-20k (Fig. 2(b)), and from 0.1 to 1 mg/ml for IgG (Fig. 2(c)). Similar linear ranges as for FITC-dextran-20k were found for FITC-dextrans-4k, -40k, and -70k. In our experiments, we therefore used 0.15 mg/ml sodium fluorescein, 1 mg/ml FITC-dextrans, and 0.5 mg/ml IgG to determine corresponding $P$ and $D_{eff}$.

**Determination of Microvessel Apparent Solute Permeability $P$.** Figure 3 shows the method used to determine $P$. Figure 3(a) demonstrates an x-z (cross-sectional) image after 75 s perfusion of FITC-dextran-20k. It shows that the fluorescence dye fills a microvessel lumen and spreads into the surrounding tissue. Figure 3(b) is the total intensity $I$ (arbitrary unit) in an ROI (the dashed line enclosed region in Fig. 3(a)) as a function of time. The size of the ROI was chosen to ensure that, during the time course in determining $P$, no significant dyes spread to the outside of the ROI and no significant contamination from the outside entered the ROI. Since the average distance between adjacent microvessels in the brain tissue is $\sim 40 \mu m$ [38], to avoid contamination from adjacent vessels, we selected the ROI whose cross section includes a microvessel lumen and a surrounding tissue region with a circumference 10–20 $\mu m$ around the vessel perimeter (Fig. 3(a)). Usually, we used the images taken 20–200 s after the vessel lumen was completely filled with dyes to determine $P$ for the solutes under study. When the fluorescently labeled solute was perfused into a cerebral microvessel, the fluorescence intensity in the vessel showed a step increase $I_0$. As the solute penetrated from the vessel further into the surrounding tissue, there was a further increase in the total intensity of fluorescence in the ROI. From the initial slope of the increasing intensity $(dI/dt)_0$ (Fig. 3(b)), the magnitude of the step increase $I_0$ and the vessel radius $r$, we can calculate the apparent solute permeability $P = I_0 (dI/dt)_0 r / 2$ [26]. Values were determined from one to three microvessels for each rat.

**Perfusion Rate and RBC Influence on $P$.** The injection rate of fluorescence solutions was 3 ml/min, which is the normal blood flow rate at the carotid artery for the rats [36,37]. At this rate, the blood was replaced by the fluorescent solute solution in the carotid artery. However, there was still residual blood in the cerebral microvessels, and those residual red blood cells (RBCs) would diminish the volume of the microvessel lumen available to the fluorescent molecule, resulting in an underestimate in the fluorescence intensity, $I_0$, for that luminal volume. As estimated in the appendix of Yuan et al. [27], under the perfusion rate of 3 ml/min at the carotid artery, the RBCs would account for $\sim 10\%$ volume of the dye solution in the microvessels and would thus reduce the
fluorescence intensity of the effective solution by \( \sim10\% \). The effective solution is the solution in which the solute in the solution can travel across the vessel wall. Therefore, the measured \( I_0 \) with the RBC presence was only \( \sim90\% \) of the true \( I_0 \) without the RBCs. If using 90\% \( I_0 \) instead of \( I_0 \) in the formula for determining \( P \), an \( \sim11\% \) overestimation in the measured apparent permeability \( P \) would be calculated.

Influence of Free Dye Associated With Fluorescence-Labeled Solutes on \( P \). We used FITC-labeled dextrans and Alexa Fluor® 488–labeled IgG to gain high quantum yield (ratio of the number of fluorescence photons emitted to the number of photons absorbed) with low light excitation. Because the presence of even small amounts of rapidly diffusible free dyes associated with the conjugated dextrans or IgG would lead to large overestimations of the permeability to the fluorescently labeled macromolecules, we used the same method as described before [27,29] to estimate the influence of free dye on the measured permeability to dextrans and IgG. The corrected solute permeability \( P_{\text{correct}} = [1/(1-F)] P_{\text{measure}} - [F/(1-F)] P_{\text{free dye}} \), where \( P_{\text{measure}} \) is the measured \( P \) for the fluorescently labeled solutes, \( P_{\text{free dye}} \) is the \( P \) for the free dye, and \( F \) is the intensity ratio of the free dye filtrate to original solutions of fluorescently labeled solutes. \( P_{\text{NaF}} \) was used for \( P_{\text{free dye}} \) since the molecular weight of the dyes is very close to that of NaF. In our fluorescence solutions, \( P \) was less than 0.3\% for FITC and less than 0.1\% for Alexa Fluor 488 for the FITC- or Alexa Fluor® 488–labeled solutes.

**Solvent Drag Contribution to \( P \).** The apparent permeability \( P \) measured in the current study would overestimate the true diffusive solute permeability \( P_d \) due to the coupling of solute flux with water flow (solvent drag or convection). The diffusive permeability \( P_d \) was calculated by the following equations [29,39,40]:

\[
P = P_d \frac{P_e}{\exp(P_e) - 1} + L_p(1 - \sigma)\Delta P_{\text{exp}}
\]

\[
P_e = \frac{L_p(1 - \sigma)\Delta P_{\text{exp}}}{P_d}
\]

where \( P \) is the measured apparent permeability, \( P_e \) is the Peclet number across the vessel wall, \( L_p \) is the hydraulic conductivity of the microvessel, \( \sim2.0 \times 10^{-9} \) cm/s/cm H2O (see below), \( \sigma \) is the reflection coefficient of the microvessel to the solute, and \( \Delta P_{\text{exp}} \) is the effective filtration pressure across the microvessel wall, obtained from

\[
\Delta P_{\text{eff}} = \Delta P - \sigma_{\text{dye-solute}} \Delta \pi_{\text{dye-solute}} - \sigma_{\text{albumin}} \Delta \pi_{\text{albumin}}
\]

where \( \Delta P \) and \( \Delta \pi \) are the hydrostatic and oncotonic pressure differences across the microvessel wall, respectively, and the superscript dye-solute can be either FITC-dextran or Alexa Fluor-IgG. No data have been reported for the reflection coefficients of rat cerebral microvessels to the test solutes. Therefore, \( \sigma_{\text{dextran}} \) and \( \sigma_{\text{IgG}} \) for rat cerebral microvessels were estimated based on previous studies [27] according to the following molecular sizes: \( \sigma_{\text{dextran}} \) was assumed to be 0.98, 0.95, 0.93, 0.90, and 0.88 \( \sigma_{\text{IgG}} \) for the free dye, \( \sigma_{\text{dextran}} \), and \( \sigma_{\text{IgG}} \) in the cerebral microvessel were estimated based on previous studies [27] according to the following molecular sizes: \( \sigma_{\text{dextran}} \) was assumed to be 0.98, 0.95, 0.93, 0.90, and 0.88. \( \Delta \pi_{\text{albumin}} \) was 3.6 cm H2O for 10 mg/ml BSA, and \( \Delta \pi_{\text{albumin}} \) was estimated to be 0.94 [41]. The hydraulic conductivity \( L_p \) used in calculating \( P \) was \( 2.0 \times 10^{-9} \) cm/s/cm H2O, which was an estimate from \( L_p \) measured from in vivo frog pial microvessels (2.0 \( \times 10^{-9} \) cm/s/cm H2O) [42], from isolated arterioles in rat brain (5.9 \( \times 10^{-9} \) cm/s/cm H2O) [43], and from isolated rat cerebral vein (2.0 \( \times 10^{-10} \) cm/s/cm H2O) [44].

**Determination of Effective Solute Diffusion Coefficient \( D_{\text{eff}} \) in Brain Tissue.** Figure 4 illustrates the determination of effective solute diffusion coefficient \( D_{\text{eff}} \) in brain tissue from the collected images. \( D_{\text{eff}} \) was determined by fitting the temporal and spatial intensity curves by a mathematical model for solute transport in the tissue space [26].

\[
\frac{\partial C_t}{\partial t} = D_{\text{eff}} \left( \frac{\partial^2 C_t}{\partial r^2} + \frac{1}{r} \frac{\partial C_t}{\partial r} \right) - \chi u \frac{\partial C_t}{\partial r} \tag{4}
\]

where \( C_t(t,r) \) is the concentration of solutes in the tissue space, \( D_{\text{eff}} \) is the effective diffusion coefficient of solutes in tissue, and \( r \) is the distance from the vessel center. \( \chi \) is the retardation coefficient of a solute in the tissue, estimated as 0.1–1 for solutes under study [45]. \( u \) is the interstitial fluid velocity in brain tissue. The Peclet number \( P_e \) in the tissue is [46]

\[
P_e = \frac{\chi L_p}{D_{\text{eff}}} \tag{5}
\]

Here, \( L_p \) is the characteristic length for the solute transport pathway, which is the mean half distance (\( \sim20 \) μm) between adjacent...
of $10^{-6} \sim 10^{-8}$ cm$^2$/s, $P_{\text{eff}}$ was calculated as in the order of $10^{-5} - 10^{-4}$, which is much smaller than 1. Thus, the convection part can be neglected in Eq. (4). Equation (4) becomes

$$\frac{\partial C_t}{\partial t} = D_{\text{eff}} \left( \frac{\partial^2 C_t}{\partial r^2} + \frac{1}{r} \frac{\partial C_t}{\partial r} \right)$$

(6)

The boundary conditions for Eq. (6) are

- at the vessel wall $r = a$ \( P(C_{\text{lumen}} - C_t) = D_{\text{eff}} \frac{\partial C_t}{\partial r} \)
- midway between adjacent vessels $r = b$ \( \frac{\partial C_t}{\partial r} = 0 \)

The initial condition is

$$t = 0, \quad C_t(0, r) = 0$$

(9)

where $C_{\text{lumen}}$ is the solute concentration in the vessel lumen and $P$ is the microvessel solute permeability.

Solving the above Eq. (6) using MATLAB, we obtained the theoretical solute tissue concentration profiles $C_t(t, r)$. To obtain the measured $C_t(t, r)$, eight straight lines were drawn from the center of a vessel lumen to a distance $\sim 20 \mu$m from the vessel wall in the tissue space (see Fig. 4(a)), the averaged intensity from these eight directions was approximated as the measured $C_t(t, r)$, which was plotted in Fig. 4(b) (green lines, $r_t = 0$ is at the vessel wall). The $D_{\text{eff}}$ in the tissue was determined by the best curve fitting of the model predictions (black lines in Fig. 4(b)) to the measured profiles [26]. The best fit for dextran-20k was $D_{\text{eff}}/D_{\text{free}} = 0.145$ in the brain tissue for this experiment.

### Data Analysis and Statistics

Data were presented as means ± SD unless otherwise specified. One-way ANOVA was used to test for significant differences in $D_{\text{eff}}/D_{\text{free}}$ between solutes of various sizes, and two-way ANOVA was applied to between-group data to test for significant differences between permeability values measured on the cerebral microvessels (current study) and those on the pial microvessels (previous study). Significance was assumed for probability levels $p < 0.05$.

### Results and Discussion

**Solute Permeability $P$ of the Blood-Brain Barrier.** A summary of the cerebral microvessel permeability $P$ to various-sized solutes is shown in Table 1. The third column shows the measured apparent solute permeability $P$, the fourth shows the permeability $P$ corrected for the influence of RBCs (≈11% overestimation), and the fifth column includes corrections for the influence of both RBCs and free dye. Free dye contributions determined as described in the Material and Methods would lead to an overestimation of 0.6%, 2.0%, 3.4%, 3.6%, and 2.1%, respectively, in $P_{\text{dextran-4k}}$, $P_{\text{dextran-20k}}$, $P_{\text{dextran-40k}}$, $P_{\text{dextran-70k}}$, and $P_{\text{IgG}}$. In the

### Table 1

<table>
<thead>
<tr>
<th>Solutes</th>
<th>No. of Vessels</th>
<th>$P$ (Measured) ($\times 10^{-7}$ cm/s)</th>
<th>$P_{\text{RBC}}$ (Corrected for RBC) ($\times 10^{-7}$ cm/s)</th>
<th>$P_{\text{RBC and Free Dye}}$ (Corrected for RBC and Free Dye) ($\times 10^{-7}$ cm/s)</th>
<th>$P_{\text{RBC, Free Dye, and Solvent Drag}}$ (Corrected for RBC, Free Dye, and Solvent Drag) ($\times 10^{-7}$ cm/s)</th>
<th>$P_{\text{RBC and Free Dye}}/P_{\text{RBC}}$ (Corrected for RBC and Free Dye)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>8</td>
<td>14.59 ± 4.03</td>
<td>12.99 ± 3.59</td>
<td>12.99 ± 3.59</td>
<td>12.92 ± 3.59</td>
<td>0.9945</td>
</tr>
<tr>
<td>Dextran-4k</td>
<td>9</td>
<td>6.16 ± 0.96</td>
<td>5.48 ± 0.85</td>
<td>5.45 ± 0.86</td>
<td>5.41 ± 0.86</td>
<td>0.9919</td>
</tr>
<tr>
<td>Dextran-20k</td>
<td>18</td>
<td>1.75 ± 0.34</td>
<td>1.55 ± 0.30</td>
<td>1.52 ± 0.30</td>
<td>1.49 ± 0.30</td>
<td>0.9791</td>
</tr>
<tr>
<td>Dextran-40k</td>
<td>11</td>
<td>1.37 ± 0.26</td>
<td>1.22 ± 0.23</td>
<td>1.18 ± 0.23</td>
<td>1.15 ± 0.23</td>
<td>0.9778</td>
</tr>
<tr>
<td>Dextran-70k</td>
<td>13</td>
<td>1.30 ± 0.20</td>
<td>1.16 ± 0.18</td>
<td>1.12 ± 0.18</td>
<td>1.11 ± 0.18</td>
<td>0.9965</td>
</tr>
<tr>
<td>IgG</td>
<td>7</td>
<td>0.54 ± 0.14</td>
<td>0.48 ± 0.12</td>
<td>0.47 ± 0.12</td>
<td>0.46 ± 0.12</td>
<td>0.9940</td>
</tr>
</tbody>
</table>

Values are mean ± SD; RBC: red blood cell.

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We note, however, that, while we carefully chose the size of the free-dye influence is greater than 97% (last column in Table 1).

In the sixth column, we calculated the diffusive solute permeability $P_d$ to various solutes. Due to a very low hydraulic conductivity of the BBB, the contribution from the solvent drag to the apparent solute permeability is negligible, since the ratio of the diffusive permeability $P_d$ to the apparent permeability corrected for the RBC and free-dye influence is greater than 97% (last column in Table 1). We note, however, that, while we carefully chose the size of the ROI and the timing for the $P$ measurement for different solutes, some degree of dye contamination from undetectable surrounding vessels may be inevitable.

Figure 5 demonstrates the measured apparent solute permeability $P$ as a function of solute size. We can see that, although dextran-20k, -40k, and -70k have a large difference in molecular weight, their BBB permeability only differs by less than 35%. However, the much smaller solutes, such as NaFl and dextran-4k, have higher BBB permeability compared to medium and large solutes. These results suggest that the BBB is highly selective to medium and large solutes but less selective to small solutes.

Comparison of $P_d$ of Cerebral Microvessels With That of Pial Microvessels. Figure 6 compares the diffusive permeability $P_d$ of rat cerebral microvessels 100–250 μm below the pia mater (current study) and $P_d$ of pial microvessels [27]. $P_d$ of cerebral microvessels is comparable to those of pial microvessels to dextrans-4k, -20k, -40k, and -70k, as well as IgG ($p > 0.29$). However, $P_d$ of cerebral microvessels to the smallest solute (fluorescein) was only about half of that of pial microvessels ($p < 0.0001$). Our results suggest that pial microvessels at the brain surface are a good model for investigating BBB transport of relatively large molecules. In Fig. 6, we also compared our permeability data with those measured using intravital microscopy [10] for pial microvessels of male adult SD rats. They are comparable to each other, indicating that the BBB permeability is similar in different genders of the same species and may also be similar in different species. If so, the measured BBB permeability in rats could be useful as a first approximation of that in humans, since a completely noninvasive method to measure the BBB permeability in human brain is not available at present.

<table>
<thead>
<tr>
<th>Solute</th>
<th>Stokes Radius (nm)</th>
<th>$D_{eff}$ at 37°C (×10^{-7} cm²/s)</th>
<th>$D_{free}$ at 37°C (×10^{-7} cm²/s)</th>
<th>$D_{eff}/D_{free}$ No. of Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>0.45</td>
<td>33.20 ± 4.66</td>
<td>72.80</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>Dex-4k</td>
<td>1.4</td>
<td>4.42 ± 0.88</td>
<td>23.00</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>Dex-20k</td>
<td>2.3</td>
<td>1.31 ± 0.26</td>
<td>11.30</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Dex-40k</td>
<td>3.0</td>
<td>0.89 ± 0.29</td>
<td>7.77</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>Dex-70k</td>
<td>3.6</td>
<td>0.59 ± 0.18</td>
<td>5.29</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>IgG</td>
<td>5.2</td>
<td>0.47 ± 0.13</td>
<td>4.10</td>
<td>0.11 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SD.

Effective Solute Diffusion Coefficient $D_{eff}$ in Brain Tissue and Comparison With Previous Studies. Effective solute diffusion coefficients $D_{eff}$ of sodium fluorescein, FITC-dextrans, and IgG were summarized in Table 2. From Tables 1 and 2, we can see that, while the absolute values of $D_{eff}$ vary by two orders of magnitude for solutes ranging from 0.45 to 5.2 nm radius, $D_{eff}/D_{free}$ varies to a much smaller extent. The $D_{eff}/D_{free}$ of small-solute sodium fluorescein was significantly different from all other solutes ($p < 0.0001$), and that of dex-4k was also significantly different from those of dex-20k, -40k, -70k and IgG ($p < 0.032$); however, there was no significant difference in $D_{eff}/D_{free}$ among larger molecules ($p > 0.9$), indicating that relative resistance of the brain tissue to movement of solutes in this size range is similar.

Figure 7 compares $D_{eff}$ measured using our in vivo minimally invasive perfusion and multiphoton system with those measured either in vivo or ex vivo in rat cortex (~100–300 μm below the pia mater) using on-site point-source application of the fluorescence-labeled solutes and spread observation by integrative optical imaging. $D_{eff}$ of tetramethylrhodamine- or Texas Red

031005-6 / Vol. 136, MARCH 2014 Transactions of the ASME
(TR)–labeled dextran-3k was 6.35–8.1 × 10⁻⁷ cm²/s, and \( D_{\text{eff}} \) of TR-dextran-10k, -40k, and -70k were 5.1, 0.91, and 0.75 × 10⁻⁷ cm²/s, respectively, measured in rat cortex slices ex vivo [18,19,47,48]. \( D_{\text{eff}} \) of TR-dextran-3k was 5.4 × 10⁻⁷ cm²/s, \( D_{\text{eff}} \) of TR-dextran-70k was 0.65 × 10⁻⁷ cm²/s, and \( D_{\text{eff}} \) of lactoferrin (Stokes radius 4.7 nm) was 0.58 × 10⁻⁷ cm²/s, measured in rat cortex in vivo [22,23].

In general, the \( D_{\text{eff}} \) values measured using our minimally invasive method with intact microvessels and brain tissue was smaller than those measured previously by direct application of the dye-labeled solutes to the brain tissue. The older, more direct methods may cause a local brain tissue damage and increase the \( D_{\text{eff}} \), even in live animals. For the ex vivo measurement on the tissue slice, the surgery for harvesting the brain tissue and the ex vivo environment for keeping the brain tissue in physiological conditions may be additional factors to increase the \( D_{\text{eff}} \).

**Effects of Excitation Laser.** In the current study, we used a multiphoton microscopy with excitation infrared laser of 100-fs pulse rate and 800–850-nm wavelength. Since infrared light may induce thermal injury to vessels and tissues at certain power, we thus calculated the energy per unit area of our laser, which was \( \sim 0.06 \text{ J/cm}^2 \) in the experiments. This value was much lower than the ablation threshold of 3 J/cm² for rat brain tissues [49] and \( \sim 1 \text{ J/cm}^2 \) for increasing vascular permeability [50], indicating a negligible effect of excitation laser on our measured \( P \) and \( D_{\text{eff}} \).

**Effects of Wavefront Distortion and Scattering of Brain Tissue.** Brain tissue is a turbid (not transparent) media, and the fluorescent images collected by the multiphoton microscope are distorted by the tissue-induced wavefront distortion and scattering due to the homogenous and inhomogeneous compositions of a variety of particles in the brain parenchyma [51,52]. Based on the measured point spread function by imaging fluorescent beads through fresh brain tissue slices by multiphoton microscopy [52], we corrected some of our images and determined the \( P \) and \( D_{\text{eff}} \) from the corrected images [53–56]. The detail is described in the Appendix. We found that, although the corrected images have better visual quality, the \( P \) and \( D_{\text{eff}} \) determined from the corrected images have negligible differences from those determined from the original images (Fig. 8).

In summary, we developed a new minimally invasive method by using multiphoton microscopy with longer excitation wavelength for better penetration of brain tissue to simultaneously quantify the blood-brain barrier permeability to solutes and effective solute diffusion coefficients in rat brain. This method can be applied to determine cerebral microvessel permeability and solute diffusion coefficients in brain tissue under a variety of physiological and pathological conditions. These quantified transport parameters can be used to develop better drugs, drug carriers, and strategies for brain drug delivery.

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**Appendix**

Due to the turbid nature of the brain tissue, our multiphoton microscopic images are affected by wavefront distortion and scattering [51]. One way to correct the distortion is to perform...
deconvolution with a point spread function (PSF), which is usually assumed to be Gaussian distribution [52] 
\[ P(x, z) = \frac{1}{\sqrt{2\pi\sigma_1\sigma_2}} \exp\left(-\frac{x^2}{2\sigma_1^2} - \frac{z^2}{2\sigma_2^2}\right), \]
where \( \sigma_1 \) and \( \sigma_2 \) are the standard deviations in \( x \) and \( z \) directions (see Fig. 1), respectively. In some of the collected images used to determine \( P \) and \( D_{\text{eff}} \), the corrected distortion in the following way. The image was then denoised by a smooth filter of matrix and then deconvoluted by an algorithm [54], which is extensively applied in image processing [53–56]. The \( \sigma_1 = 722 \text{ nm} \) and \( \sigma_2 = 4034 \text{ nm} \), which were used in the correction algorithm, corresponding to the full width at half maximum of 1700 nm and 9500 nm in \( x \) and \( z \) directions, respectively, which were estimated from the measured PSF by imaging fluorescent beads through the fresh brain slice by multiphoton microscopy [52].

Figure 8 shows the comparison of the original (top left) image and that corrected (top right) for the waveform distortion and scattering. The corrected image has a better visual effect. In the bottom plot, we compared the effect of the image distortions on determining the solute brain tissue diffusion coefficient \( D_{\text{eff}} \). The green lines are the curves of the averaged intensity (or concentration) over eight directions versus time (see Fig. 4(b)) from the original images, the red lines are from the corrected ones, and the black lines are the model predictions with the best-fitting \( D_{\text{eff}}/D_{\text{free}} \). After correction, the tissue concentration distribution profiles (red lines) are smoother than those (green lines) from the original image. However, the best fitting curves (black lines) for \( D_{\text{eff}}/D_{\text{free}} \) are the same. The same is true for determining the vesSEL permeability \( P \).

References


