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Small separation diffuse correlation spectroscopy for measurement of cerebral blood flow in rodents

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Abstract: Diffuse correlation spectroscopy (DCS) has shown promise as a means to non-invasively measure cerebral blood flow in small animal models. Here, we characterize the validity of DCS at small source-detector reflectance separations needed for small animal measurements. Through Monte Carlo simulations and liquid phantom experiments, we show that DCS error increases as separation decreases, although error remains below 12% for separations > 0.2 cm. In mice, DCS measures of cerebral blood flow have excellent intra-user repeatability and strongly correlate with MRI measures of blood flow (R = 0.74, p <0.01). These results are generalizable to other DCS applications wherein short-separation reflectance geometries are desired.

1. Introduction

Adequate cerebral blood flow (CBF) is critical for delivery of oxygen and nutrients necessary to maintain neuronal health and function. Abnormalities in blood perfusion are seen in numerous diseased states, including stroke [1,2], hemorrhage [3], traumatic brain injury [4,5], hypoxic-ischemia [6–8], and hypertension [9,10]. These abnormalities encompass not only significant reductions or elevations in flow, but also vital impairments in the autoregulatory and neurovascular coupling mechanisms that regulate vascular tone [11–13]. Thus, cerebral hemodynamics can serve as an informative biomarker of injury severity, progression, and mechanism.

Preclinical models provide an invaluable tool to explore mechanisms underlying cerebral blood flow regulation, in addition to enabling the development of novel therapeutic strategies that restore perfusion and improve outcome after injury. Rodent (particularly rat and mouse) models are especially appealing given the wide range of genetically modified strains available and the relatively low cost compared to other large animal models. Currently, a handful of techniques exist to enable quantification of cerebral blood flow in small animal models including autoradiography [14,15], perfusion magnetic resonance imaging (MRI) [16], laser Doppler flowmetry (LDF) [17–19], laser speckle contrast imaging (LSCI) [20,21], and micro-ultrasound [22]. While each technique certainly has its own advantages, numerous limitations remain, including the need for radioactive material (autoradiography), poor spatial resolution (LDF), low SNR (MRI), limited depth penetration (LSCI), and sensitivity to macrovascular blood flow velocity instead of microvascular blood flow (Doppler ultrasound).
Diffuse correlation spectroscopy (DCS) [23–26] is an emerging optical modality that has shown promise to non-invasively measure CBF in small animal models [27–33]. In DCS, coherent near-infrared (NIR) light multiply scatters as it diffuses through scalp, skull, and brain before detection at some distance away on the tissue surface. Scattering off moving red blood cells within the tissue leads to temporal fluctuations in the detected light intensity. These temporal fluctuations are quantified through a temporal intensity autocorrelation function, \( g_2(\tau) \); \( g_2(\tau) \) is then fit to simple analytical models, known as correlation diffusion theory [23,34], to extract a cerebral blood flow index (CBF_i). This CBF_i represents a bulk average of the microvascular flow in the “banana-shaped” region that spans from the source to the detector [23,24]. DCS offers several advantages over other blood flow modalities used in small animals described above. Most importantly, in contrast to autoradiography, laser speckle contrast imaging, and laser Doppler flowmetry, DCS measurements are noninvasive, as data acquisition does not involve animal sacrifice or even resection of the scalp or thinning of the skull, thus enabling longitudinal monitoring of cerebral blood flow within the same animal. Further, the instrumentation is relatively inexpensive (~$50k) and data can be obtained relatively quickly (<1 min) under light anesthesia or even in awake animals [31,32]. Moreover, DCS appears to be sensitive to perfusion in the microvasculature, i.e., capillaries, arterioles, and venules [26,35] in contrast to techniques like Doppler ultrasound [36] that measure flow velocity in large feeding arteries.

The correlation diffusion models used in DCS are predicated on the assumption that the source detector separation is greater than the transport mean free path, defined as the inverse of the reduced scattering coefficient [23,37]. Thus, for typical brain tissue with \( \mu_s' \sim 10 \text{ cm}^{-1} \), source detector separations >> 0.1 cm are needed to ensure the validity of these models. In humans and large animal models, this requirement is easily met, as large source detector separations of > 1 cm [38,39] are routinely employed given the depth to the cortex. Indeed, numerous validation studies performed with large source detector separations have demonstrated excellent agreement between DCS and other CBF modalities i.e. MRI [40–45], microspheres [46], perfusion CT [47,48], indocyanine green (ICG) tracers [49], and transcranial Doppler [50–53]. However, in small animal models wherein the skull and scalp are ~0.1-0.2 cm thick [54] and the brain itself is only about 1-2 cm thick, source detector separations of 0.3-1 cm are needed for depth penetration to cortical microvasculature.

Herein, we explore the validity of the diffusion approximation at small source-detector separations (<1 cm) required to probe brain tissue in small animal models. We utilize a series of \textit{in silico}, \textit{in vitro}, and \textit{in vivo} experiments to characterize the accuracy, validity, and repeatability of utilizing correlation diffusion theory to analyze DCS data taken at source-detector separations of 0.1-1 cm. Further, we explore the effects of constraining the fit of \( g_2(\tau) \) to early delay times to minimize signal contributions from photons that have traveled shorter paths and to thereby improve accuracy when employing the diffusion approximation. We note that the results from these analyses may be generalized beyond small animal cerebral blood flow studies to any application of DCS wherein the desired penetration depth dictates the use of small source-detector separations.

2. Material and methods

2.1 \textit{In silico verification using Monte Carlo simulations}

Monte Carlo simulations were used for computational verification of DCS at small (< 1 cm) source-detector separations. Monte Carlo eXtrem e (MCX) was used for all simulations [55]. For each simulation, we launched 500 million photons, which took <10 min on a GPU (NVIDIA Quadro P600). Using a 6 \times 6 \times 6 \text{ cm}^3 semi-infinite medium with anisotropic factor \( g \) of 0.9 and index of refraction \( n \) of 1.4 to mimic biological tissue, we ran a total of 784 unique simulations for 14 source-detector separations (\( \rho \)), 7 absorption coefficients (\( \mu_a \)), and 8 reduced scattering coefficients (\( \mu_s' \)) (14 separations \times 7 \( \mu_a \times 8 \mu_s' = 784 \) unique simulations).
Separations ranged from 0.1 to 2 cm (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.2, 1.5, 1.8, 2 cm), \( \mu_a \) ranged from 0.05 to 0.35 \( \text{cm}^{-1} \) in steps of 0.05 \( \text{cm}^{-1} \), and \( \mu_s' \) ranged from 3 to 15 \( \text{cm}^{-1} \) (3, 5, 7, 9, 10, 11, 13, 15 \( \text{cm}^{-1} \)). From each simulation, MCX records the total pathlength traveled and total number of random walk steps for each detected photon. Separate simulations were run for each source-detector separation to avoid absorption by a proximal detector. For each simulation, the detected normalized electric field autocorrelation function, \( g_1(\rho, \tau) \), was computed using the total momentum transfer of the detected photons [56]:

\[
g_1(\rho, \tau) = \sum_{i=1}^{N_p} \frac{e^{-K^2(\tau)_{i}}}{r_1 - r_2} - \frac{e^{-K(0)_{i}}}{r_1 - r_2},
\]

(1)

Here, \( N_p \) is the number of detected photons, \( Y_i \) is the total dimensionless momentum transfer for the \( i \)th detected photon, \( L_i \) is the total pathlength traveled by the \( i \)th detected photon, \( k_o \) is the light wavenumber in the medium \( (k_o = \frac{2\pi}{\lambda}) \), \( \lambda \) is the wavelength of light (set to 852 nm to match our experimental setup), and \( \langle \Delta r^2(\tau) \rangle \) is the mean-square particle displacement at delay time, \( \tau \) [34,56]. Here, we assumed Brownian motion of the moving scatterers such that \( \langle \Delta r^2(\tau) \rangle = 6D_B \tau \), where \( D_B \) is the Brownian diffusion coefficient [24]. For each of the 784 simulations, we computed \( g_1(\rho, \tau) \) for 6 different physiologically realistic \( D_B \) values seen experimentally (denoted below as \( D_B,\text{known} \)), ranging from \( 0.5 \times 10^{-8} \) to \( 3 \times 10^{-8} \) \( \text{cm}^2/\text{s} \) in steps of \( 0.5 \times 10^{-8} \) \( \text{cm}^2/\text{s} \). Thus, in total we simulated 4704 \( g_1(\rho, \tau) \) curves (784 simulations \( \times 6 D_B \) per simulation).

To estimate the accuracy of DCS, each simulated \( g_1(\rho, \tau) \) data set was fit for \( D_B \) (denoted \( D_B,\text{fit} \)) using the semi-infinite solution to the correlation diffusion equation [23]:

\[
g_1(\rho, \tau) = \left[ \frac{e^{-K^2(\tau)}}{r_1 - r_2} - \frac{e^{-K(0)}}{r_1 - r_2} \right],
\]

(2)

where \( K^2(\tau) = 3\mu_a(\lambda)\mu'_s(\lambda) + \mu'_s(\lambda)^2 k_o^2 \alpha \langle \Delta r^2(\tau) \rangle \), \( r_1 = \sqrt{\rho^2 + z_0^2} \), \( r_2 = \sqrt{\rho^2 + (z_0 + 2z_b)^2} \), \( z_0 = 1/\mu'_s \), \( z_b = 2(1 + R_{\text{eff}})/(3\mu'_s(1-R_{\text{eff}})) \), \( R_{\text{eff}} = 0.493 \) [57] is the effective reflection coefficient, and \( \alpha \) is the fraction of scattering events that occur from moving scatterers in the medium (assumed to be 1 for these simulations). Error in \( D_B \) for each simulated \( g_1(\rho, \tau) \) curve was defined as \( (D_B,\text{fit} - D_B,\text{known})/D_B,\text{known} \times 100\% \).

Further, because we are often interested in how blood flow changes over time, we also estimated how errors in \( D_B \) get translated to errors in relative change in \( D_B \) (\( rD_B \)). For each \( D_B \), we characterized a range of physiologically realistic relative changes in blood flow ranging from \(-90\%\) to \(200\%\) of the baseline \( D_B \) value (denoted \( D_{B,0,\text{known}} \)). Error in the relative change in \( D_B \) was estimated as \( (rD_B,\text{fit} - rD_B,\text{known})/rD_B,\text{known} \times 100\% \), where \( rD_B,\text{known} \) is the simulated relative change in \( D_B \) from \( D_{B,0,\text{known}} \) to \( D_{B,i,\text{known}} \), \( rD_B,\text{fit} = (D_{B,0,\text{fit}} - D_{B,i,\text{fit}})/D_{B,0,\text{fit}} \times 100\% \), and \( D_{B,0,\text{fit}} \) and \( D_{B,i,\text{fit}} \) denote the best fit values using the semi-infinite correlation diffusion equation solution to the simulated data from \( D_{B,0,\text{known}} \) and \( D_{B,i,\text{known}} \) respectively.

2.2 DCS acquisition and analysis

For experimental verification of DCS at small SDS reflectance geometries, we used a custom-built DCS instrument, comprised of an 852 nm long coherence length laser (iBeam smart, TOPTICA Photonics, Farmington, NY), a photon counting avalanche photodiode (SPCM-AQ4C-IO, Perkin-Elmer, Quebec, Canada), and a hardware autocorrelator board (Flex05-8ch, www.correlator.com, NJ). For all \textit{in vitro} and \textit{in vivo} experiments described herein, we
utilized a custom optical sensor consisting of a 1 mm source comprised of a tightly packed bundle of 50μm multi-mode fibers (NA = 0.66, FTIIG23767, Fiberoptics Technology, Pomfret, CT) spaced 0.6cm away from a single-mode detection fiber (780HP, Thorlabs, Newton, NJ).

Experimentally, DCS measures the normalized intensity autocorrelation function, $g_2(\tau)$. The Siegert relation [58] was used to convert this measured $g_2(\tau)$ to $g_1(\tau)$; Eq. (2) was then used to fit $g_1(\tau)$ for $\alpha_{DB}$. Fits were constrained to $g_1(\tau) > 0.01$. Secondary analysis constrained fits to $g_1(\tau) > 0.4$ to explore the effect of this cutoff on DCS validity [59]. In vitro, we assume $\alpha = 1$, and in vivo, we define $\alpha_{DB}$ to be the cerebral blood flow index, or CBFi, as this parameter characterizes the dynamics of the interrogated tissue. These fits require knowledge of the absorption and scattering properties of the medium at 852 nm. In vitro, we measured $\mu_a$ and $\mu_s'$ of the homogenous liquid phantoms using multi-distance (1.5, 2, 2.5, and 3 cm) frequency-domain near infrared spectroscopy (Imagent, ISS, Champaign, Illinois) as in [41]. In vivo, we assumed values of $\mu_a = 0.25 \text{ cm}^{-1}$ and $\mu_s' = 7 \text{ cm}^{-1}$ for mouse brain [60].

2.3 In vitro phantom verification

For benchtop verification of DCS, we compared the measured $\alpha_{DB}$ at a large source-detector separation (1.5 cm) where diffusion theory holds true to a small separation (0.6 cm) commonly used in rodent brain studies [31,32]. Two (3 L) homogenous liquid phantoms of differing reduced scattering coefficients were used. Phantoms were held in a (28 × 17 × 14 cm$^3$) container and consisted of 20% Intralipid (Fresenius Kabi, Lake Zurich, IL) and India Ink (Higgins, Chartpak, MA) for scattering and absorption contrast, respectively. The measured optical properties at 852 nm of each phantom were $\mu_s' = 10 \text{ cm}^{-1}$, $\mu_a = 0.12 \text{ cm}^{-1}$ and $\mu_s' = 4.6 \text{ cm}^{-1}$, $\mu_a = 0.12 \text{ cm}^{-1}$, respectively. To manipulate $\alpha_{DB}$ in these phantoms, temperature was varied slowly from 6 to 70°C. DCS measurements were made periodically in 2–3°C increments. At every temperature increment, acquired measurements were repeated three times at each source-detector separation, and the measurement order for 0.6 and 1.5 cm was randomized. Temperature differences between the two source-detector separation measurements were < 0.5°C at any given temperature increment.

2.4 In vivo verification against ASL-MRI

We validated DCS-measured CBFi against blood flow measured by Arterial Spin Labeling (ASL) MRI in a mouse model [61,62]. All animal procedures were approved by Emory University Institutional Animal Care and Use Committee and followed the NIH Guidelines for the Care and Use of Laboratory Animals. Adult male C57BL/6 mice (n = 9) were induced with 4.5% isoflurane for 45 s and maintained at 1.8% isoflurane (1 L/min, 70% nitrous oxide, 30% oxygen mixture) in a stereotaxic holder for the duration of the study. Body temperature and respiration rate were monitored and maintained at 36.5°C and 75-90 breaths per minute, respectively. Depilatory cream was used to remove hair from the scalp to ensure adequate signal-to-noise for DCS measurements. Once stabilized, animals were placed in the MRI scanner and imaged for ~40 minutes (see Section 2.4.1), at which time the mouse was immediately taken out of the scanner for DCS acquisition to ensure measurements are made under approximately the same physiological conditions.

For DCS measurements, the optical sensor was gently placed over the intact scalp such that the source and detector fibers aligned with the parasagittal plane (source fiber approximately at bregma −0.1, 0.3 cm mediolateral). DCS data were acquired for five seconds over each hemisphere; measurements were repeated three times per hemisphere and averaged to yield a mean CBFi for the right and left hemisphere. DCS data were rejected from analysis if the intensity (i.e. photon count rate) was less than 30 kHz, or if the coefficient of variation (COV) was greater than 20%, where COV was defined as the standard deviation / mean CBFi across multiple repetitions on a given hemisphere.
We note that in order to obtain a wide range of blood flow for DCS validation, a closed head injury model (described in depth in Buckley et al. [32]) was used in a subset of n = 2 mice to produce a mild traumatic brain injury 4 hours prior to imaging. This injury paradigm has been shown to decrease blood flow by ~30% at 4 hours post-injury.

2.4.1 ASL-MRI acquisition and analysis

MRI measurements were performed using a 9.4 T/20 cm horizontal bore Bruker magnet, interfaced to an Avance console (Bruker, Billerica, MA, USA). A two-coil actively decoupled imaging set-up was used (a 2 cm diameter surface coil for reception and a 7.2 cm diameter volume coil for transmission). Sagittal T2-weighted anatomical reference images were acquired with a RARE (Rapid Acquisiton with Refocused Echos) sequence for the slice localization of interest. Slice location was chosen to encompass the sagittal plane that spanned the midpoint between the DCS source and detector. Imaging parameters were as follows: TR = 1800 ms, Eff.TE = 45 ms, RARE factor = 8; field of view (FOV) = 38.4 × 19.2 mm², matrix = 256 × 128, Avg = 4, slice thickness (thk) = 0.7 mm. Perfusion images were acquired with the vendor provided ASL protocol using a flow-sensitive alternating inversion recovery (FAIR) technique and a RARE sequence. Imaging parameters were as follows: TR = 18000 ms, Eff.TE = 20 ms; RARE factor = 8, FOV = 26.6 × 16 mm², matrix = 64 × 40, Avg = 1, thk = 1 mm, number of TIR (inversion recovery time) = 8 (30, 200, 600, 1200, 2400, 4000, 6000, 10000 ms). Paired images were acquired alternately with and without arterial spin labeling. Anatomical T2-weighted images were acquired on the same slice of the perfusion image with a RARE (Rapid Acquisition with Refocused Echoes) sequence Imaging parameters were as follows: TR = 4000 ms, Eff.TE = 40 ms, RARE factor = 8, Avg = 4, The FOV, matrix, and slice thickness were the same as the perfusion image.

In the FAIR technique [62,63], two inversion recovery images are acquired (non-slice-selective and slice-selective inversion pulses) in an interleaved fashion. The longitudinal magnetization after both a selective (ss) and nonselective (ns) inversion pulse can be defined as:

\[
M_{ss} = M_0 \left(1 - 2e^{-\frac{TI}{T_1}}\right), \tag{3}
\]

\[
M_{ns} = M_0 \left(1 - 2e^{-\frac{TI}{T_1}}\right). \tag{4}
\]

Here \(M_0\) is the fully relaxed longitudinal magnetization of the tissue-water proton, \(T_1\) is the longitudinal magnetization time of tissue-water proton, \(T_{1*}\) is the apparent longitudinal relaxation time, and \(TI\) is the inversion time. From the non-slice-selective and slice-selective inversion pulses, the values for \(M_{ns}\), \(M_{ss}\), and \(TI\) are known; thus, we solved for \(T_1\), \(T_{1*}\), and \(M_0\) through systems of equations. After obtaining the values for \(T_1\) and \(T_{1*}\), it is possible to estimate blood flow, \(f\) (mL/min/100g) with the equation [62,63]:

\[
\frac{1}{T_{1*}} = \frac{1}{T_1} + \frac{f}{\delta}, \tag{5}
\]

where we assume the tissue-to-blood partition coefficient, \(\delta\), is 90 mL/100g [64].

To compare flow measured with ASL-MRI to CBF, measured with DCS, ROIs were chosen to roughly encompass the region of tissue probed by DCS (~0.2 cm into the cortex) while balancing the need for sufficient ASL SNR within the ROI. We selected two five-sided polygon ROIs that encompassed the right and left superior quadrants to either side of the midline using the roipoly.m function in MATLAB. ROIs were chosen such that two of the legs had dimensions of 0.48 and 0.28 cm. Average \(M_{ns}\) and \(M_{ss}\) within each ROI was computed for all TI, and Eqs. (3) and (4) were used to compute a mean ROI flow for comparison to DCS.
2.5 Small separation DCS repeatability

Given the known effects of sensor position (i.e., regional inhomogeneity under the tissue surface) and pressure (i.e., changes in scalp flow during compression) on DCS repeatability at large separations [52,65], we next estimated small separation DCS repeatability through both an in vivo mouse model and an in vitro liquid phantom model, which served as a control experiment.

For in vitro repeatability assessment, DCS measurements were made by manually holding the optical sensor over two predefined locations marked on a container that held a liquid phantom consisting of 1.58 μm diameter microspheres suspended in water ($\mu_s'(852\ nm) = 5.68\ cm^{-1}$, Bangs Laboratories Inc.). Three users acquired three repetitions per location, and the resultant fit for $D_B$ were averaged to yield a mean and standard deviation $D_B$ value at each location.

For in vivo repeatability assessment, adult C57BL/6 mice (n = 10) were induced with 4.5% isoflurane for 1 minute and maintained at 1.5% isoflurane (1L/min, 70/30 nitrous oxide/oxygen mixture). After stabilization, three users each took three DCS measurements on each hemisphere by gently resting the sensor on the animal’s head. Measurements were acquired once a day for three days. To account for blood flow measurement changes among users due to time differences between measurements and anesthesia depth, the order between the three users was randomized per hemisphere. The measurements on each hemisphere from each user, were averaged to yield a mean (standard deviation) cerebral blood flow index per hemisphere per animal.

2.6 Statistical analysis

For in vitro phantom verification, Lin’s concordance correlation coefficient (CCC) [66] was used to quantify the agreement between the Brownian diffusion coefficient measured at a small source detector separation ($D_B,0.6cm$) to that of a large source detector separation ($D_B,1.5cm$). Further, as a graphical means for assessing agreement between small and large separation measurements, Bland-Altman plots of the difference versus the mean of the small and large separation data were generated [67].

For ASL-MRI and DCS in vivo validation, linear regression analysis was performed to quantify any significant relationship between blood flow measurements from DCS and ASL-MRI. Pearson’s correlation coefficient, $R$, was used to quantify the extent to which a linear model explains variability in the data. All regression analyses were performed using R statistical software (R Foundation for Statistical Computing). Hypotheses tests and associated p-values were two sided. Statistical significance was declared for p-values < 0.05.

Finally, an intraclass correlation coefficient (ICC) [68,69] was used to assess both intra- and inter-user reliability of small separation DCS measurements. We used a two-way random effects model for ICC that treats users as random samples from the population of users and the experimental model (i.e. mouse and liquid phantom) as random samples from the experimental population. ICC was calculated using absolute agreement as a more conservative estimate of repeatability of DCS. ICC within a single user or across multiple users indicates the amount of experimental error that can be attributed to variation specific to the experimental model relative to the total variation in the measurements. Ideally, we would want both intra-user and inter-user measurements to have an ICC close to 1, indicating that the total variation is solely due to experimental model variability. Based off published values, ICC values greater than 0.75 are classified as excellent repeatability, ICC between 0.6 and 0.74 are classified as good repeatability, between 0.4 and 0.59 are classified as fair, and less than 0.4 are classified as poor reliability [69].
3. Results

3.1 In silico Monte Carlo verification

Representative Monte Carlo simulated $g_1(\rho, \tau)$ curves along with the best fit lines using the semi-infinite solution to the correlation diffusion equation are shown in Fig. 1(a),(b). As source detector separation decreased, the deviation of the diffusion approximation fit from the simulated $g_1(\rho, \tau)$ becomes more apparent. Fits improve when constraining $g_1(\rho, \tau)$>0.4. At typical optical properties seen in brain tissue ($\mu_a'=10$ cm$^{-1}$, $\mu_s=0.2$ cm$^{-1}$), the error in estimating $D_B$ using the diffusion approximation was <12% at source-detector separations greater than 0.2 cm (Fig. 1(c)). By constraining fits to where $g_1(\rho, \tau)$>0.4, the error is slightly reduced to <10% at source-detector separations > 0.2 cm (Fig. 1(d)). As expected, at large source-detector separations (> 1.5 cm) the error in $D_B$ was small (<1%). The error in $D_B$ was independent of $D_B$ known; however, error was heavily influenced by $\mu_a$ and $\mu_s'$. To demonstrate the effects of $\mu_a$ and $\mu_s'$ on the error in $D_B$, Fig. 1(e) depicts error in $D_B$ known (1x10$^{-8}$ cm$^2$/s) at a source-detector separation of 0.6 cm, as a function of absorption and scattering. The highest error in $D_B$ (~28%) was observed at the highest simulated $\mu_a$ (0.35 cm$^{-1}$) and lowest simulated $\mu_s'$ (3 cm$^{-1}$); error then decreases as $\mu_a$ decreases and $\mu_s'$ increases. Again, by constraining $g_1(\rho, \tau)$ fits to $g_1(\rho, \tau)$>0.4, the effects of $\mu_a$ and $\mu_s'$ on the error in $D_B$ is slightly reduced to ~25% (Fig. 1(f)). Finally, although errors in absolute $D_B$ made at small separations are on the order of 5-10%, errors in relative change in $D_B$ are negligible (<0.5%, Fig. 1(g),(h)).
3.2 In vitro phantom verification

Figure 2(a) displays representative $g_2(\rho, \tau)$ curves measured on the same liquid phantom at both 0.6 and 1.5 cm source detector separations. While the correlation diffusion theory fit for $g_2(\rho, \tau)$ at 1.5 cm matches well with the measurement, the fit for $g_2(\rho, \tau)$ at 0.6 cm slightly diverges from the measurement. This fit at 0.6 cm improved when constrained to only fit
Despite these small errors in the fit at 0.6 cm, the estimated $D_{B,0.6cm}$ and $D_{B,1.5cm}$ show a good agreement for both phantoms (Fig. 2(b),(c)). For fits constrained to $g_2(\rho, \tau) > 1.005$, Lin’s concordance coefficients, CCC (95% confidence interval), was 0.98 (0.97, 0.99) and 0.83 (0.71, 0.90) for the two phantoms, indicating strong agreement between $D_{B,0.6cm}$ and $D_{B,1.5cm}$. Bland-Altman plots of this data reveal that all but one data point fall within 1.96 standard deviations of the mean percent difference between the 0.6 and 1.5 cm measurements, providing conformational graphical representation of this strong agreement (Fig. 2(d),(e)).
3.3 Strong correlation between ASL-MRI and DCS

Of the 9 mice imaged, 3 were excluded due to poor T1 image quality. Of the remaining 6 mice, one left hemispheric DCS measurement was discarded based on the exclusion criteria described in Section 2.4. Thus, a total of 11 hemispheric data points from n = 6 mice are included for the final comparison between ASL and DCS. Fig. 3(a) shows a representative anatomical image of the ASL slice taken in the midbrain. The red polygon denotes the region of interest used to quantify an average hemispheric cerebral blood flow. Across all 6 mice, we observed a highly significant correlation between ASL-MRI measures of cerebral blood flow and DCS measures of CBF measured at 0.6 cm (R = 0.74, p = 0.0088, Fig. 3(b)).

(a) (b)

Fig. 3. (a) Anatomical image of ASL-MRI mouse with one ROI (red) used to obtain a perfusion value of CBF concurrent with the region that DCS measures. (b) Cerebral blood flow index measured by DCS versus cerebral blood flow measured by ASL-MRI in both healthy (n = 4, 7 hemispheric data points, red) and concussed mice (n = 2, 4 hemispheric data points, cyan). Data from left and right hemisphere were treated as independent samples. Black line denotes line of best fit and grey shaded region denotes 95% confidence interval. Error bars represent the standard deviation.

3.4 Small separation DCS is highly repeatable

Repeatability measurements taken with a 0.6 cm source-detector separation on a liquid phantom revealed an excellent inter-user ICC value of 97%. For mice, the ICC within each user (N = 3 users) was 86%, 81%, and 90%; these values are categorized as excellent. However, the inter-user ICC in mice was only 51%, classified as fair repeatability between users (Fig. 4).
4. Discussion

In this study, we explore the validity of diffuse correlation spectroscopy in small separation reflectance geometries. A key assumption in correlation diffusion theory is that the source-detector separation must be large in relation to the average random photon walk step size ($\ell^* = 1/\mu_s^*$). This assumption is necessary such that the photon scattering angles will be sufficiently randomized at the point of detection [23]. In transmission geometries, Kaplan et al. demonstrated that DCS works best for slab thicknesses $> 10\ell^*$, although in the case of highly anisotropic scattering (as is the case in biological tissue) DCS can work for slab thicknesses as small as $3\ell^*$ [37]. However, this same rigorous assessment of the breakdown of DCS has not been performed in reflectance geometries that are most commonly used in biological applications. While a handful of publications have begun to use small (<1 cm) DCS reflectance geometries to study blood flow in the mouse brain [32,70], neonatal rat brain [31], mouse and rat hindlimbs [71], mouse tumors [72], mouse bone graft [73,74], skin flap [75,76], this work is the first to our knowledge that explores the accuracy of these reflectance measurements in a regime where DCS may break down.

Through Monte Carlo simulations, we found that the correlation diffusion approximation does indeed provide a suitable model for small separation reflectance DCS data. For typical absorption and scattering properties of brain tissue, diffusion theory yielded errors in $D_B$ of $<$12% for source detector separations as small as 0.2 cm. As expected, this error decreased with increasing separation. By constraining fits to those photons associated with shorter $\tau$ and thus longer pathlengths by thresholding $g_1(\rho,\tau) > 0.4$, this error in the estimation of $D_B$ was reduced to $<$10% for all tested source-detector separations $> 0.2$ cm. Moreover, we found that these errors effectively cancel out when estimating relative changes in $D_B$, suggesting that short-separation DCS can be used to accurately track longitudinal changes in cerebral blood flow (assuming the influence of extracerebral layers are negligible).

We next expanded our error quantification beyond the typical optical properties of brain. We found that the error in $D_B$ increased substantially as $\mu_a^*$ decreased and $\mu_s^*$ increased. This trend was expected, given that the validity of the diffusion approximation is predicated on nearly isotropic radiance (where isotropy is achieved when $\mu_s^* >> \mu_a$ [23]). However, even when $\mu_s^* = 3$ cm$^{-1}$ and $\mu_a = 0.35$ cm$^{-1}$, the error in $D_B$ at 0.6 cm remained below $<$25% if the fit for $g_1(\rho,\tau)$ was constrained to $> 0.4$. This increase in error with decreasing $\mu_s^*$ was confirmed in vitro through our liquid phantom studies that revealed the error in $D_B$ at $\mu_s^* = 10$ cm$^{-1}$ was roughly half the error seen at $\mu_s^* = 4.6$ cm$^{-1}$.

Although the error in using the diffusion approximation to fit DCS data at source detector separations needed to probe rodent brain is $<$12% for typical brain optical properties, this
error may not be negligible for studies in which the effect size of interest is small. As mentioned above, constraining $g_2$ fits to shorter $\tau$ may serve to improve accuracy, as these photons have traveled deeper through tissue [59] and become fully randomized before detection. Indeed, we observed a reduction in the error of $D_B$ when constraining fits to early delay times, although this constraint did not fully ameliorate the error. Future work can explore the depth sensitivity of small separation DCS for a range of delay time constraints, as in Selb et al. [59]. Alternatively, to further improve accuracy, one could employ a lookup table-based Monte Carlo approach wherein $g_2$ curves are simulated for a wide range of $D_B$ values, and then the measured $g_2$ data are fit to this simulated $g_2$ database [77,78].

We confirmed our in silico and in vitro results through translation to an in vivo mouse brain study. As expected, we observed a significant positive correlation between DCS blood flow index measured at 0.6 cm source-detector separation and ASL-MRI measures of absolute cerebral blood flow in mice. We note that the strength of this correlation was likely influenced by several limitations in the experimental design and measurements. First, DCS and ASL-MRI data were not acquired simultaneously because the optical sensor was not MRI compatible, thus introducing the possibility for physiological variation in the time that lapsed between the DCS and ASL measurements (~5 min). Second, DCS measurements represent a bulk average of the banana-shaped region that spans from source to detector, resulting in extracerebral (skull, scalp, cerebrospinal fluid) signal contributions that could be significant. Third, ASL data is subject to low SNR given the small brain size of mice and the long acquisition time (~20 min for a single blood flow image) and is highly susceptible to motion artifacts caused by respiration. Finally, we had to assume a constant value for $\mu_a$ and $\mu_s'$ for all animals. As discussed below, this assumption can lead to significant error in $D_B$ if the true optical properties deviate significantly from assumed values. As Diop et al. [49] demonstrated, measuring individual animal $\mu_a$ and $\mu_s'$ would improve DCS estimations. Despite these limitations, the significant correlation between ASL and DCS suggests that DCS can provide a sensitive index of cerebral blood flow.

Finally, in the mouse brain, we found excellent repeatability in the DCS measured blood flow index within a given user, although repeatability across different users was only classified as fair. Because inter-user repeatability in the well-controlled phantom experiment was excellent, we attribute inter-user variability in the mouse to both the subtle differences in the positioning of the sensor or possibly to the extent of applied sensor pressure that a particular user typically exerts [65]. Whatever the reason, given the excellent intra-user repeatability seen in vivo, we recommend that the same operator acquire DCS measurements in the case of longitudinal experiments wherein the same animal may be measured at multiple time points.

One main limitation in the estimation of an absolute blood flow index with DCS is the need for a priori knowledge of $\mu_a$ and $\mu_s'$ in the sample of interest. In reality, $\mu_a$ and $\mu_s'$ are often not known and must be assumed from literature-reported values (as we did in our in vivo mouse studies). Unfortunately, if these assumed values are inaccurate, they can induce substantial errors in $D_B$ [79]. However, frequency- or time-domain near-infrared spectroscopy techniques that are commonly used in large animal or human studies to quantify $\mu_a$ and $\mu_s'$ may not be accurate in the rodent head geometry, as the photon diffusion equation that governs these techniques also breaks down at small separations. Hallacoglu et al. [80] demonstrated that $\mu_a$ and $\mu_s'$ can be measured in the adult rat using multi-distance frequency domain NIRS, although they note that data diverged significantly from diffusion approximation for source-detector separations < 0.8 cm. Thus, for mice or neonatal rats, alternative approaches must be employed to estimate tissue optical properties. Our laboratory has developed a Monte Carlo simulation based inverse algorithm to estimate absorption and scattering properties using small separation frequency domain near-infrared spectroscopy that may provide a suitable alternative to estimate $\mu_a$ and $\mu_s'$ in mice and young rats [77].
Although our primary application of small source-detector separation DCS was focused on the rodent brain, we emphasize that the results of this study are generalizable towards other applications. There is a plethora of biological applications that may benefit from a perfusion monitor for tissue at depths of ~0.1-0.4 cm, wherein other optics-based flow techniques like laser Doppler flowmetry or laser speckle contrast imaging do not provide sufficient depth penetration. These applications include studies of perfusion in the skull and scalp in humans, burn wounds in humans where primary tissue damage occurs in epidermis and dermis [81,82], preclinical models of skeletal muscle [71], skin flap/graft monitoring [73,83], and breast cancer models [72].

5. Conclusion

We demonstrate that the semi-infinite solution to the correlation diffusion equation provides a suitable approximation to fit DCS data obtained in a reflectance geometry at small (0.2-1 cm) source detector separations with excellent \textit{in vivo} intra-user repeatability. Absolute blood flow index estimation is subject to <12% error at these separations, and error in the estimation of the relative change in blood flow is < 0.5%. These results suggest that DCS may be used to accurately estimate cerebral blood flow in small animal models, as well as other biological applications where a depth penetration >0.1 cm is desired.

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