Exploring diazepam’s effect on hemodynamic responses of mouse brain tissue by optical spectroscopic imaging

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Abstract: In this study, a simple duel-optical spectroscopic imaging apparatus capable of simultaneously determining relative changes in brain oxy-and deoxy-hemoglobin concentrations was used following administration of the anxiolytic compound diazepam in mice with strong dominant (Dom) and submissive (Sub) behavioral traits. Three month old mice (n = 30) were anesthetized and after 10 min of baseline imaging, diazepam (1.5 mg/kg) was administered and measurements were taken for 80 min. The mouse head was illuminated by white light based LED’s and diffused reflected light passing through different channels, consisting of a bandpass filter and a CCD camera, respectively, was collected and analyzed to measure the hemodynamic response. This work’s major findings are threefold: first, Dom and Sub animals showed statistically significant differences in hemodynamic response to diazepam administration. Secondly, diazepam was found to more strongly affect the Sub group. Thirdly, different time-series profiles were observed post-injection, which can serve as a possible marker for the groups’ differentiation. To the best of our knowledge, this is the first report on the effects of an anxiolytic drug on brain hemodynamic responses in mice using diffused light optical imaging.

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OCIS codes: (170.0110) Imaging systems; (170.3880) Medical and biological imaging; (170.4580) Optical diagnostics for medicine; (170.6510) Spectroscopy, tissue diagnostics.

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Depressive and anxiety disorders are clinically problematic with a majority of cases showing multiple recurrences and frequent progression to the chronic stage [1, 2]. Unfortunately, our limited understanding of the neurobiology of these disorders hinders the development of new therapies and therapeutic strategies [3]. Although treatment success rates for depressive and anxiety disorders have increased over the past two decades, they still remain low [4]. Therapeutic compounds’ efficacy is assessed mainly based on behavioral changes in both preclinical and clinical studies, while objective physiological parameters of treatment’s success have yet to be developed. Functional MRI (fMRI) studies of psychotropic drugs administration as well as electroencephalograms (EEGs) mapping show neural activity
patterns that are congruent with the behavioral changes [5–7]. The effect of diazepam has been investigated recently with the help of positron emission tomography (PET) and computed tomography (CT) [8]. Diazepam, a member of the benzodiazepine family, acts through modulation of GABAergic neurotransmission by producing anxiolytic, sedative or/and anticonvulsive effects [9]. It is used in treatment of disorders characterized by anxiety, agitation, tremors, delirium, seizures, as well as hallucinations resulting from alcohol withdrawal [10, 11]. It is also used to relieve muscle spasms in some neurological diseases, and for sedation during surgery [9]. While the above mentioned techniques have greatly advanced our ability to understand brain function and the effects of drug administration on brain physiology, each of them suffer from certain constraints (expensive and not portable, require physical restraint, are complex in structure, etc.) or do not provide quantitative information regarding chromophore concentration levels, such as oxyhemoglobin (HbO2) and deoxyhemoglobin (Hbr). Hence, the influence of the psychotropic drugs on brain hemodynamics needs to be more clearly understood.

The limitations and drawbacks of conventional neuro-imaging techniques have led to extensive interest in optical diagnosis techniques such as diffuse optical imaging (DOI). DOI has gained great interest due to its unique properties, such as the ability to assess real-time changes in the concentration of hemoglobin in finer resolution than the aforementioned methodologies [12]. DOI techniques have been successfully applied in medicine and biomedical research to monitor normal and abnormal tissue structure and function. Particularly, DOI has been used in clinical diagnostics and management in different fields of neuroscience such as neurology, neurosurgery, psychiatry, and rehabilitation [13–17]. Within this context: (1) biological tissues are relatively transparent to light in the near infrared (NIR) range between 650 and 950nm [18]; (2) there is high correlation between brain activities to changes in brain tissue’s optical properties dependent upon hemodynamics [19]; (3) neural activity can be detected through the skull surface [20–23]; and (4) optical techniques have some unique properties: being noninvasive and inexpensive, offer unsurpassed high spatiotemporal resolution, are portable and relatively low-cost, require minimal patient restraint, etc.. These traits offer advantages in comparison to conventional functional neuroimaging methodologies.

This manuscript describes an attempt to monitor variations in brain hemodynamics in response to drug administration. This is obtained by applying a white light source to the scalp followed by the acquisition of a series of images of the diffuse reflected light filtered at 470 and 650 nm, which is mainly sensitive to HbO2 and Hbr concentrations, respectively. We analyzed these images to examine the spatiotemporal characteristics of a set of hemodynamic parameters. We observed distinct hemodynamic reactions between groups of animals in response to diazepam over time. It should be stressed that following imaging, for example at 470nm, the achieved data will include absorption from both HbO2 and Hbr. However, since HbO2 is approximately ten-fold more absorptive than Hbr, we can consider the acquired image to be mainly influenced by HbO2. One can reduce the mutual influence between the two using frequency or time domain approaches [12].

Several optical methods have been widely applied in medicine and biomedical research to monitor structure and function in living organisms and patients [12, 24, 25]. The dynamic response of brain tissue hemoglobin levels to drug administration based on NIR spectroscopy was reported [26–28]. However, to the best of our knowledge, the application of highly cost effective optical imaging to investigate the effects of anxiolytic drugs on brain hemodynamics related to rodent’s behavior has yet to be reported. Hence, we first hypothesized that continuous-wave (CW) diffuse reflectance optical imaging can be utilized in studies of mice to detect and monitor altered brain hemodynamic in response to anxiolytics. Secondly, we anticipated that the changes in tissue constituent can be measured simply and inexpensively with CW light.

Our hypothesis was tested on mice with genetic predisposition to strong dominant and submissive behavioral phenotype [29, 30]. These animals were developed based on dominant-submissive relationship model used for psychotropic drug screening [31–34]. Our recent
studies demonstrated that these animals differentially react to acute diazepam administration: while submissive animals showed obvious anxiolytic and sedative response to diazepam administration dominant mice demonstrated paradoxical response that was manifested in anxiogenic and hyperactive behavior [30]. Thus, we hypothesized that differential behavioral response of these animals to diazepam may be also reflected in the changes in hemodynamic parameters.

2. Material and methods

2.1. Animal and experimental protocol

This study was carried out using a protocol approved by the Institute Animal Care and Use Committee of Ariel University. The populations of selectively bred dominant (Dom) and submissive (Sub) mice (n = 30, males, mean weight = 45 gr, 3 month old) used in this study are descendants of the outbred Sabra strain. Animal’s behavioral features of dominance and submissiveness are confirmed using dominant-submissive relationship (DSR) test. Details of the behavioral procedure and the breeding have been published previously [29]. In addition to the Dom and Sub groups, background wild-type (WT) Sabra mice [35] were used as a control group.

A solution of Xylazine (Xylazine 20 Inj. Kepro B.V., Netherlands) was used in ratio 1:6 to Ketamine (Ketanest. Fort Dodge, USA), further diluted 1:1 with Saline (NaCl, 0.9%) for anesthesia kept the mouse motionless throughout the experiment. Mice received intraperitoneal injection according their weight (6 µl of total solution per gram of mouse). The depth of anesthesia was ascertained by pinching of the toes or tail and by monitoring rate of breathing. Hair causes a multiple scattering which affect the captured photon density and contributes to a large fraction of the overall light. These cause image degradation and blurring which reduce the ability to observe the head surface by the camera. In order to obtain a clear image, hair was removed to increase efficacy of light transmission and reflection [36, 37]. A folded heating plate was placed under the mouse to keep the body temperature at a constant level of ~34°C and a thermocouple rectal probe (YSI) was inserted to measure core body temperature. Other physiological (systematic) parameters such as heart rate and arterial oxygen saturation (SpO2) were continuously monitored independently through a veterinary pulse oximeter (Nonin 8600, Plymouth, MN, USA) attached to the forelimb.

The experimental protocol consisted of a 10 min baseline reflectance images recording of the head, injection of diazepam (1.5 mg/kg) without interrupting the optical monitoring, and 80 min of reflectance measurements post diazepam injection. After the experiments, the animals were euthanized by carbon dioxide (CO2).

2.2. Experimental setup

Figure 1 illustrates the imaging system. Collimated white light based LED’s technology shining the head (~30mm in diameter) at an incident angle of ~30 deg off the normal to the head surface. The diffusely reflected light (intrinsic optical signals) from the head, which embodies tissue physiological properties, is split equally into two directions (channels) using 50:50 plate beamsplitter (BS) (Thorlabs, BSW10R). Since in mouse the layers of the head are optically thin and highly transparent to light, we consider the diffuse reflected signals to have originated from the brain itself. Each channel consists of different 10nm wide optical bandpass filter (BPF, Thorlabs) of 470nm and 650nm, respectively and 14-bit CCD camera (GuppyPRO F-013B, Allied Vision Technology, 480 × 640 pixels resolution, Germany) equipped with a zoom lens system (Computar, 75-150 mm, f/2.8, New York, USA). In that way, the diffusely reflected light is first filtered accordingly and then recorded simultaneously by the CCD cameras which view the same region through the BS. Imaging acquisition, synchronization, and data processing are achieved using software implemented in the Matlab platform (Version 2010a, The MathWorks, Inc., Natick, Massachusetts) controlled via personal computer (Intel Core, E6750). The CCD gain and image integration time were manually adjusted during imaging. The setup is similar to one presented previously [38, 39]
but differs in its light source engine and configuration, channel filtering, data processing, and application.

**Fig. 1. Sketch of the dual-imaging setup. BPF, band pass filter; BS, beam splitter; L, lens (f = 100 mm). Representative images with the selected ROI are shown for each channel. ROI size: 50 × 60 pixels correspond to ~5mm × 5mm. The same ROI is observed simultaneously on both cameras.**

### 2.3. Hemodynamics quantification

The major approach we implement in this work to measure the changes in hemodynamics is based on the fact that in the NIR range (650-950nm, known as optical window) the primary light absorber compounds (chromophores) in biological tissue are oxygenated (HbO₂) and deoxygenated (Hbr) hemoglobin molecules [40]. In addition, differences in HbO₂ and Hbr concentrations can be calculated at least from two wavelength measurements [41]. The relative low absorption factors in this optical band enable one to see changes in HbO₂ and Hbr deep within tissue (up to several centimeters), including the head [42]. Depending on their state of oxygenation, the absorption spectra of hemoglobin’s are different across the optical window i.e., the absorption coefficient of HbO₂ differs from that of Hbr along most of the NIR spectrum. Specifically, around the wavelength of \( \lambda = 650\text{nm} \), Hbr absorb light with five times the absorption coefficient of HbO₂ while at \( \lambda = 470\text{nm} \), the majority of the absorption arises from HbO₂, ~ten-fold more absorptive than Hbr [43]. Several neuroimaging platforms rely on this distinctive behavior of oxy-and deoxyhemoglobin. For instance, in optical intrinsic signal imaging (OISI) technique, a 630nm illuminates the cortex to emphasize changes in Hbr during whisker stimulation [44]. Different laboratories use a light source shining at isosbestic wavelengths such as 570, 800nm, etc., to observe variations in total hemoglobin concentration or blood volume [40, 45]. Application of the same concept is also applied in brain injury studies such as: spreading depression [46] and epilepsy [47]. Hence, by analyzing the spatiotemporal changes in diffuse reflected light (alternative absorption) at 650nm (BP1, Hbr sensitive) and 470nm (BP2, HbO₂ sensitive), one can simultaneously derive the variations in hemoglobin oxygenation following drug administration. It should be pointed out that at the selected wavelength of 470nm the absorption peak is higher, which in turn can affect the penetration depth \( \delta \); absorption increase \( \rightarrow \delta \) decrease. Nonetheless, since in mouse the layers are relatively thin (~micron) it is assumed that light reaches the brain surface. In addition, brain tissues are spatially heterogeneous that can affect the captured signal (partial-volume effects). Since at 470nm the majority of the absorption arises from oxy-hemoglobin (HbO₂) it can be said that variations in the detected signal are generated mainly from HbO₂. We assume here that these variations in light absorption were due to different responses to drug administration by distinct brain regions. In addition, spectral analysis to estimate the hemoglobin concentration changes can be done through the modified Beer-Lambert law taking into account the path length factors assumed in the conversion from
optical density changes to hemoglobin changes (Eq. (2) [48]). Finally, based on the hemoglobin state, further physiological parameters such as tissue oxygen saturation level (StO$_2$ = [HbO$_2$ / (HbO$_2$ + Hbr)] × 100) and cerebral blood volume (CBV = HbO$_2$ + Hbr) can be derived; CBV is proportional to total hemoglobin concentration (THC). Generally speaking, CBV serves as a marker for cerebral blood flow (CBF), relying upon the assumption that the concentration of red blood cells (hematocrit level) remains constant following drug administration. Both are parametrically related following the power-law relationship: CBV = CBF$^\Phi$ where 0<$\Phi$<0.4 [49, 50]. Also, the influence of various absorbing molecules at physiologic concentrations lying with these wavelengths such as melanin, melanosomes, collagen, cytochrome-c oxidase, carotenes, etc., is insignificant in comparison to HbO$_2$ and Hbr [51–54]. In addition, water, which is significant chromophore in the optical window, has negligible absorption below 700nm compared to hemoglobin [55].

2.4. Data analysis

Raw images from the camera were processed by in-house software developed in Matlab. Prior to data analysis, the collected diffuse raw images were first normalized to overcome the nonlinearity of the camera quantum efficiency at the above wavelengths and second, filtered (fspecial function in MATLAB) and averaged to reduce physiological fluctuations such as pulsations of the brain due to respiration, heartbeat, and arterial oscillations, and to eliminate high frequency noise originated from the camera during recording. For measurement of small fluctuations in reflectance, a simple algorithm based on a ratio that provides diffuse reflectance signal change in terms of fractional change from baseline was implemented. Consecutive ten baseline raw images were captured and averaged together (I$_o$) and image at the $i$th time point (I$_i$) was subtracted and divided by I$_o$ based on the following calculation: $\Delta R = \Delta I / I_o$ where $\Delta I = I_i - I_o$, yielding a reflectance image in dimensionless units. $\Delta R$ represents the mean percent change in reflectance signal and it is inversely proportional to light absorption; reflectance increase absorption decrease and vice versa. Since optical intrinsic signal are usually very small or noisy we calculated the changes in light reflectance from concomitantly collected images in order to emphasize signal intensity and to improve signal-to-noise ratio. Because we were not concerned with real-time display of results, data analysis was performed off-line.

2.5. Statistical analysis

Statistical significance of difference between pre- and post-drug administration and between different mice groups was evaluated using one-way ANOVA with Bonferroni-corrected post-hoc $t$-test analysis (GraphPad Prism software). Statistical differences are shown as * at $p < 0.05$, ** at $p < 0.01$, and *** at $p < 0.001$. All data are given as mean value ± standard deviation.

3. Results and discussion

Figure 2 shows a plot of the time courses of $\Delta R$ calculated over the ROI (dashed boxed region appears in Fig. 1) both before and after i.p. administration of diazepam at different wavelengths for the different animal groups. The mean value for each time point was taken from ten mice for the $\lambda$ = 470nm (HbO$_2$) and 650nm (Hbr). The error bars (standard deviation) represent the variation in the calculated mean reflectance over ROI. These variations can be explained by the individual differences (e.g., behavior, weight, level of dominancy), unexpected variances (breathing, grasping, uncontrolled general activity, etc.), partial volume effect, etc. As can be seen, in contrast to the WT (Fig. 2(a)), Dom (Fig. 2(b)) and Sub (Fig. 2(e)) groups produce opposing patterns of $\Delta$HbO$_2$ and $\Delta$Hbr. Moreover, while $\Delta$HbO$_2$ and $\Delta$Hbr levels of WT mice remain relatively constant overtime, these values for Dom and Sub mice deviated from the baseline. Among the two, $\Delta$Hbr in the Sub group strongly deviated from baseline (~3 times than $\Delta$HbO$_2$). As shown in Fig. 2, the levels of $\Delta$Hbr in Sub mice were lower prior to diazepam administration in comparison to Dom and
WT groups. Even more, these levels of $\Delta H_{br}$ were reduced in Sub mice in comparison to WT and Dom groups following injection. This is an interesting observation suggesting that submissiveness may correlate with elevated hemoglobin oxygenation levels. The strong changes in $\Delta H_{br}$ seen among Sub mice in comparison to other groups may be supported by our recent study showing differential effects of diazepam on these mice [30]. In general, the changes of $\Delta H_{br}$ concentration are of particular clinical interest in most of the optical modalities as it is closely linked to the BOLD contrast used in MRI [56]. It is worth noticing that following injection, the $\Delta H_{bO_2}$ gradually increased over time in both Dom (Fig. 2(b)) and Sub (Fig. 2(c)) mice, while no change in $\Delta H_{bO_2}$ for WT (Fig. 2(a)) were observed.

Fig. 2. Changes in brain hemoglobin concentrations over time following diazepam administration in: (a) wild-type (WT, $n=10$), (b) dominant (Dom, $n=10$) and (c) submissive (Sub, $n=10$) animals. The change in diffuse reflectance $\Delta R$ in 470nm is proportional to $H_{bO_2}$ while at 650nm to $H_{br}$. Data is presented as mean plus standard deviation (error bar) normalized to baseline. The response in diffuse reflectance to diazepam differed markedly between the three groups.
In addition, in the Dom and Sub groups, the maximum change in reflectance for both wavelengths is reached about ~50 minutes post-injection, while in the WT no such change over time is observed. Importantly, each group displays a unique time-response profile which may serve as a possible marker to distinguish between the groups. This hypothesis remains to be tested thoroughly in the future with larger animal populations. In all recordings presented in Fig. 2, the measurement noise level was also evaluated by calculation of the coefficient of variation (CV) defined as the ratio of standard deviation and the mean value. The CV was found to be lower than 1.5%.

Figure 3(a) presents the average of $\Delta$HbO$_2$ and $\Delta$Hbr over time for different groups derived from Fig. 2. Significant differences between $\Delta$HbO$_2$ (Fig. 3(b)) and $\Delta$Hbr (Fig. 3(c)) among the groups with $p<0.001$ were found. A differential diazepam-induced oxy/deoxy hemoglobin response (increase in $\Delta$Hbr and a decrease in $\Delta$HbO$_2$) was seen among the distinct animal populations. We assume that these changes may result from differential modulation of the GABAergic system induced by diazepam upon genetically distinct animal groups. This in turn may cause changes in hemoglobin fractions. As mentioned above, from the clinical point of interest, the information presented here on $\Delta$Hbr is the basis of the BOLD signal in fMRI studies.

Time traces for the changes in additional physiologically important parameters (THC, StO$_2$) for each group were calculated as mentioned earlier (subsection 2.3) and are plotted in Fig. 4. The percent change in THC in the Sub group (Fig. 4(a)) was found to be more than 5% lower from the rest; the THC in both Dom and WT groups did not remarkably change between pre and post injection. At the same time, StO$_2$ in the Sub group (Fig. 4(b)) increased gradually over time respective to its pre-injection, reaching the range of the other groups.
addition, StO$_2$ and THC levels in Sub mice were significantly lower in comparison to others pre-injection. We may speculate that these changes in StO$_2$ and THC over time may indicate that Sub animals’ glucose metabolism may differ from that of the other groups. This intriguing phenomena should be further elucidated in future study.

![Graph of THC and StO2 levels over time for different groups](image)

Fig. 4. Time course of changes in (a) THC and (b) StO$_2$ parameters extracted from the raw data of HbO$_2$ and Hbr for the wild type (WT), dominant (Dom) and submissive (Sub) mice group. Each data point represents the mean ± standard deviation (error bar). These graphs highlight the difference of the submissive group in comparison to others.

Figure 5 shows averages THC and StO$_2$ levels of each animal group, before and after diazepam treatment. While no significant differences were found in THC levels pre-injection (Fig. 5(b)) and in StO$_2$ levels post-injection (Fig. 5(e)) between Dom and WT groups, all other compared groups show highly significant differences (Fig. 5(a)-5(e)). As mentioned above, diazepam induced distinct hemodynamic changes among mice groups. Taken together, the above observations demonstrate that the proposed optical platform used here may be valuable in neuroscience rodent research for the evaluation of drug administration’s effects as well as changes in brain hemodynamics in pathological and physiological conditions.
As in other two-dimensional optical imaging modalities, the majority of the signals in the current technique are from the superficial layers of the brain. Only a portion of the signals travel deeply and return to the surface, particularly when the signal passes through intact scalp. Thus, our results are an average of signals from different head tissue layers (partial volume effect), which may impact hemodynamics imaging. In future studies, simultaneous imaging and diffuse reflectance based source-detector fiber optics configuration [57] is highly recommended to validate drugs’ effects on hemodynamics.

4. Conclusions
In summary, we have showed the effects of the anxiolytic compound diazepam on mouse brain hemodynamic parameters through intact scalp using a relatively simple and inexpensive diffuse-reflectance optical imaging. Our previous study demonstrated that diazepam differentially influenced animals with behavioral features of dominance or submissiveness.
In this study, by using an optical setup we showed that in agreement with behavioral alterations, diazepam caused distinct changes the brain hemodynamics of Dom and Sub animals. The differences in $\Delta$Hbr levels observed here are in agreement with previously reported studies based on fMRI [58]. We believe that these responses in hemodynamics are mainly due to changes in the delivery of oxygen and glucose to the brain and in metabolite clearance caused by diazepam. Since these first-reported data are preliminary, the long-term study of larger animal populations is required to further understand the differential effects of diazepam among the groups. In addition, further study is required to understand the diffuse reflectance behavior in terms of light scattering, especially taking into account that light scattering is influenced by brain activation [59, 60] and by oxygen saturation [61]. Since the apparatus used in this study is simple, portable and easy to implement, it can be used concurrently and routinely for drug activity screening to understand the interrelationships between neural, hemodynamic, and other brain activities. Furthermore, the ability to measure changes in hemodynamics with low-cost optical components grant the current method an advantage of cost-effectiveness.