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Functional Transcranial Doppler Ultrasound for Monitoring Cerebral Blood Flow

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TITLE:

Functional Transcranial Doppler Ultrasound for Monitoring Cerebral Blood Flow

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KEYWORDS:

functional transcranial Doppler, fTCD, transcranial Doppler, TCD, breath-holding index, BHI, breath-hold acceleration index, BHAI

SUMMARY:

Functional transcranial Doppler ultrasound complements other functional imaging modalities, with its high temporal resolution measurement of stimulus-induced changes in cerebral blood flow within the basal cerebral arteries. This Methods paper gives step-by-step instructions for using functional transcranial Doppler ultrasound to perform a functional imaging experiment.

ABSTRACT:

Functional transcranial Doppler ultrasound (fTCD) is the use of transcranial Doppler ultrasound (TCD) to study neural activation occurring during stimuli such as physical movement, activation of tactile sensors in the skin, and viewing images. Neural activation is inferred from an increase in the cerebral blood flow velocity (CBFV) supplying the region of the brain involved in processing sensory input. For example, viewing bright light causes increased neural activity in the occipital lobe of the cerebral cortex, leading to increased blood flow in the posterior cerebral artery, which supplies the occipital lobe. In fTCD, changes in CBFV are used to estimate changes in cerebral blood flow

With its high temporal resolution measurement of blood flow velocities in the major cerebral arteries, fTCD complements other established functional imaging techniques. The goal of this Methods paper is to give step-by-step instructions for using fTCD to perform a functional imaging experiment. First, the basic steps for identifying the middle cerebral artery (MCA) and optimizing the signal will be described. Next, placement of a fixation device for holding the TCD probe in place during the experiment will be described. Finally, the breath-holding experiment, which is a specific example of a functional imaging experiment using fTCD, will be demonstrated.

INTRODUCTION:

In neuroscience research, it is often desirable to monitor real-time brain activity noninvasively in a variety of environments. However, conventional functional neuroimaging modalities have limitations that impede the ability to capture localized and/or rapid activity changes. The true (non-jittered, non-retrospective) temporal resolution of functional magnetic resonance imaging (fMRI) is currently of the order of a few seconds[1], which may not capture transient hemodynamic changes linked to transient neural activation. However, although functional near-infrared spectroscopy (fNIRS) has high temporal resolution (milliseconds) and reasonable spatial resolution, it can only probe hemodynamic changes within the anterior cerebral cortex and cannot provide information about changes taking place in the larger arteries supplying the brain. In fTCD—classified as a neuroimaging modality—"imaging" refers to the dimensions of time and space, rather than the two dimensions of space that are more familiar in an "image". fTCD provides complementary information to these modalities by providing a high temporal resolution (typically 10 ms) measurement of hemodynamic changes at precise locations in the large vessels of the basal cerebral circulation. As with other neuroimaging modalities, fTCD may be used for a variety of experiments such as studying lateralization of cerebral activation during language-related tasks[2–4], studying neural activation in response to various somatosensory stimuli[5], and exploring neural activation in various cognitive stimuli such as visual tasks[6], mental tasks[7], and even tool production[8].

Although fTCD offers several advantages for use in functional imaging, including low cost of equipment, portability, and enhanced safety (compared to Wada test[3] or positron emission tomography [PET] scans), operation of a TCD machine requires skills obtained by practice. Some of these skills, which must be learned by a TCD operator, include the ability to identify various cerebral arteries and the motor skills necessary to precisely manipulate the ultrasound probe during the search for the relevant artery. The goal of this Methods paper is to present a technique for using fTCD to perform a functional imaging experiment. First, the basic steps for identifying and optimizing the signal from the MCA, which perfuses 80% of the cerebral hemisphere[9], will be listed. Next, placement of a fixation device for holding the TCD probe in place during the experiment will be described. Finally, the breath-holding experiment, which is one example of a functional imaging experiment using fTCD, will be described, and representative results will be shown.

PROTOCOL:

All human subject research was performed in accordance with the Institutional Review Board of the University of Nebraska-Lincoln, and informed consent was obtained from all subjects.

1. Locating the MCA signal by freehand TCD

NOTE: "Freehand" TCD refers to operation of TCD with a handheld transducer to find a CBFV signal before beginning an fTCD experiment.

1.1. Setting TCD parameters

1.1.1. Keep the power at a reasonably high value (e.g., 400 mW) during the initial search for the MCA. Once the MCA signal is located, reduce the power as much as possible while still maintaining a "good" signal (see step 2.2.7).

NOTE: Using a reasonably high power during the initial search does not violate the "As Low As Reasonably Achievable" (ALARA) principle of exposure to acoustic radiation because higher power will allow the MCA signal to be discovered more quickly[10].

1.1.2. Set the sample volume to 8–12 mm during the initial search for the MCA signal. If the signal is difficult to find, increase the gate size to increase the intensity of the signal, but note that this may incorporate the signal from one or more nearby arteries into the signal from the MCA.

1.1.3. Set the gain at a medium level, with the goal of "keeping background noise at a minimum, but present"[10].

1.1.4. Set the high-pass filter cutoff (normally termed "threshold") to 50–150 Hz.

1.1.5. If the subject is an adult, set the depth to 50 mm, which is the average mid-point depth of the M1 segment of the MCA[10] (**Figure 1**).

NOTE: This setting will be discussed in more detail in subsequent steps. Depth settings for children are given in **Table 1**.

[Place Figure 1 Here]

1.2. Locating the temporal window

NOTE: The temporal window, also called the transtemporal acoustic window, is a part of the skull where the bone is thinnest[11], thus allowing transmission of low-frequency ultrasound energy through the cranium (**Figure 2**).

[Place Figure 2 Here]

1.2.1. For infants and small children, locate the temporal window just in front of the ear (the "intertragal space") and above the rostral edge of the zygomatic arch, which can be easily felt under the skin.

1.2.2. For teenagers and young adults, locate the temporal window via any of the subwindows.

NOTE: The posterior subwindow usually provides the best signal (**Figure 2**).

1.2.3. For adults aged 30 years or older, locate the temporal window just in front of the ear.

NOTE: The acoustic window decreases in size as people age due to increasing porosity of the bone of the cranium, causing some older people to have a very limited temporal window[12]. In such individuals, bilateral insonation of the MCA is sometimes impossible.

1.3. Applying the transducer

1.3.1. Apply enough ultrasound gel to cover the surface of the transducer.

NOTE: When placed on the head, the gel should cover sufficient space to maintain a seal between the scalp and the Doppler probe's surface, thus preventing signal interruption from air coupling underneath the probe's surface.

1.3.2. Alert the subject that the gel may feel cold (if at room temperature).

1.3.3. Place the transducer on the temporal window, which was located in section 1.2.

1.4. Searching for the MCA

1.4.1. After placing the transducer on the scalp, search for the MCA signal, which will generally be located slightly anterior (forwards) and rostral (towards the head) from the location of the initial transducer scalp placement[10].

1.4.2. If the TCD spectral signal is not immediately obvious, adjust the angle of the transducer while keeping it in the same location relative to the scalp. Slowly angle the probe from rostral to caudal (towards feet) and posterior to anterior.

NOTE: **Figure 3** shows two spectra taken from the same position, but at different angles.

[Place Figure 3 Here]

1.4.3. If a signal is still absent after performing step 1.4.2, check the color M-mode display for flow in the MCA at different depths (indicated by red coloring). Increment or decrement the signal depth in 5 mm steps and search as described in step 1.4.2. If flow is visible in M-mode but not in the Doppler spectrum, increase or decrease the depth until the flow signal is visible in the Doppler spectrum.

1.4.4. If a satisfactory signal is still not obtained, move the transducer to a nearby position on the scalp, which is slightly more anterior, and repeat steps 1.4.1–1.4.3.

1.4.5. When an optimal MCA signal is obtained, note the depth and maximum velocity.

1.4.6. Using a washable makeup pen, place a mark on the scalp (trace part of the transducer edge) where the optimal signal was found.

1.5. Searching for the bifurcation

NOTE: Finding the bifurcation of the internal carotid artery (ICA) is important to help confirm that the MCA is the artery being monitored. This step should be performed on both sides if bilateral monitoring will be performed, as the bifurcation may not be at the same depth on both sides.

1.5.1. Increase the depth until the signal from the bifurcation of the ICA into the MCA and ACA is noted (**Figure 4**), typically at a depth of 51–65 mm[10].

[Place Figure 4 Here]

1.5.2. Search for the optimum bifurcation spectral signal using the procedure described in step 1.4.2. Always strive for the highest-velocity spectral signal possible[10].

1.5.3. When an optimal bifurcation signal is obtained, note the depth of the bifurcation.

1.5.4. For bilateral monitoring, repeat sections 1.1–1.4 and steps 1.5.1–1.5.3 on the other side of the head.

1.6. Relocating the MCA after placing a fixation device

NOTE: For fTCD experiments, it is necessary to monitor CBFV for 10–90 min or longer. Therefore, a fixation device (**Figure 5**) is crucial to provide stability.

1.7. Placing the fixation device

1.7.1. By visual inspection, adjust the fixation device (**Figure 5**) to the subject's approximate head size.

[Place Figure 5 Here]

1.7.2. Alert the subject before placing the headset on his or her head. Place the headset on the subject's head.

NOTE: If the subject has long or thick hair, it may be necessary to tie the subject's hair back, depending on the fixation device being used.

1.7.3. Adjust the fixation device's fit, and ask the subject if the device is too tight.

NOTE: The device should be tight enough that it does not move when bumped slightly, but loose enough that the subject is not uncomfortable.

1.8. Locating the MCA signal

1.8.1. Loosen the mechanism of the fixation device holding the transducer in place (e.g., loosen

the mechanism, shown in in **Figure 5**, by turning a knob counterclockwise) so that the transducer can move freely.

1.8.2. Alert the subject before applying gel to the transducers (which should already be in place from section 2.1), and that the gel may be cold (if it has been stored at room temperature).

1.8.3. Apply enough ultrasound gel to the transducer to cover the face of the transducer.

1.8.4. Adjust the fixation device so that the transducer is located over the top of the mark made in step 1.4.6.

1.8.5. Search for the optimal MCA spectral signal using the procedure described in steps 1.4.1–1.4.3. Always strive for the highest-velocity spectral signal possible[10].

NOTE: When compared to freehand TCD, the optimal depth at which the MCA is located using the fixation device may differ slightly (at most 1–2 mm) from the depth for the freehand device. This is because the fixation device may hold the transducer slightly further away from the scalp while still maintaining a coupling gel seal.

1.8.6. When the optimal MCA spectral signal is found, tighten the mechanism of the fixation device to lock the transducer in place. Note the depth and all other settings.

1.8.7. Decrease the power (see step 1.1.1) as much as possible while still maintaining a spectral envelope that traces the maximal velocity accurately.

1.8.8. For **bilateral monitoring**, repeat steps 2.1.1–2.2.7 on the other side.

1.9. Performing a breath-hold maneuver

NOTE: This section is given as an example of a functional experiment that may be performed using the experimental setup described in section 1 and section 1.6.

1.9.1. Perform all steps described in section 1 and section 1.6.

1.9.2. Begin recording on the TCD software.

1.9.3. Breathe normally for 3 min to achieve a good baseline recording, and allow CBFV to stabilize from any previous experiments or stimuli.

1.9.4. Count down slowly from three. On the count of one, ask the subject to begin breath-holding following a normal inspiration[13].

NOTE: The subject should not inhale deeply, as this would decrease carbon dioxide in the lungs and decrease the likelihood of observing the increase in CBFV due to cerebrovascular reactivity. The subject should also avoid performing a Valsalva maneuver, in which intrathoracic pressure is

substantially increased against a held inspiration[14].

1.9.5. Place a marker in the TCD recording to signify the start of breath-holding.

1.9.6. Have the subject hold their breath for 30 s, or until they are no longer comfortable holding their breath.

1.9.7. When the subject inhales, place a marker in the TCD recording to signify the end of breath-holding.

1.9.8. Continue monitoring CBFV using TCD and recording for at least 30 s following the end of breath-holding to ensure that CBFV returns to baseline values.

REPRESENTATIVE RESULTS:

Figure 3 shows sample Doppler spectra and color M-modes from the midpoint of the M1 segment of the MCA. **Figure 3A,B** were taken at the same position on the scalp, but at different angles. Note how a very small change in angle, *without changing the contact position on the scalp*, can greatly improve Doppler signal strength, as shown by the higher-intensity yellow coloring of the spectrogram in **Figure 3B**. Note also that the M-mode in **Figure 3B** shows two arteries (blue and red, corresponding to the ACA and MCA, respectively).

Figure 4 shows a sample Doppler spectrum and M-mode from the bifurcation of the ICA into the ACA and MCA. Note the overlapping red- and blue-shaded regions in the M-mode image denoting the MCA and ACA, respectively. Also note the symmetry of the Doppler spectral waveform when comparing flow towards the transducer (positive) with flow away from the transducer (negative).

Figure 6 shows sample spectra and M-mode images from different time points in the breath-hold maneuver. **Figure 6A** shows the baseline TCD spectrum and M-mode at the beginning of breath-holding. Note the mean velocity of 56 cm/s. **Figure 6B** shows the TCD spectrum and M-mode at the end of breath-holding. Note that the mean velocity has now increased to 70 cm/s. **Figure 6C** shows the TCD spectrum and M-mode after the end of breath-holding. Note the undershoot in velocity below baseline values, with the mean dropping to 47 cm/s. Note that the ACA is visible as flow away from the transducer (blue) in the Doppler spectra.

Figure 7 shows the entire breath-holding experiment. Note that the envelope remains elevated for approximately 15 s after breath-holding ends, falls to values lower than those at the beginning of breath-holding for ~20 s, and then finally recovers to baseline values. Note that the ACA is visible as flow away from the transducer in the Doppler spectrum.

Figure 6 and **Figure 7** display good signal intensity in the MCA portion of the TCD spectrum (the MCA is represented by the positive velocities); note how the white line which represents the envelope follows the TCD spectrum very accurately when the spectrum is bright. The spectra of **Figure 6** and **Figure 7** could be improved by decreasing the monitoring depth by 5–10 mm so that the ACA portion of the TCD spectrum would not be visible (the ACA is represented by negative

velocities) and by changing the scale of the vertical axis in the TCD spectrum to run from approximately -100 cm/s to 100 cm/s, which would allow maximum velocity sampling of the TCD spectrum in the vertical direction.

Figure 8 shows examples of bilateral TCD spectra and M-modes suitable for bilateral fTCD. **Figure 8A** and **Figure 8B** demonstrate acceptable, but not optimal, bilateral spectra and M-modes. Note how the gain is higher in **Figure 8A** (left MCA) than in **Figure 8B** (right MCA) to compensate for the weaker signal, and how the envelope quality in **Figure 8A** is slightly poorer than in **Figure 8B**. Also note how the maximum velocity at systole in **Figure 8A** is slightly lower than in **Figure 8B**. By contrast, note how the two spectra in **Figure 8C** and **Figure 8D** are very similar in terms of settings, including depth, gain, power, and sample volume, and how the spectral waveforms on both sides have similar maximum velocities and shapes. To address this, it is recommended that the spectrum from the left MCA be consistently placed in the left window and the spectrum from the right MCA in the right window, especially for experiments involving lateralization of blood flow. This is also consistent with TCD usage in a medical context.

FIGURE AND TABLE LEGENDS:

Figure 1: Representation of the circle of Willis and the major arteries of the cerebral circulatory system. The bifurcation of the ICA into the ACA and MCA is marked with a black circle. The M1 segment of the MCA is shown. This figure has been modified from [24]. Abbreviations: ACA = anterior cerebral artery; Bif. = bifurcation; ICA = internal carotid artery; MCA = middle cerebral artery.

Figure 2: The transtemporal window (marked by the dashed ellipse), zygomatic arch (arrow), and subwindows[11]. (**A**) Frontal subwindow. (**B**) Anterior subwindow. (**C**) Middle subwindow. (**D**) Posterior subwindow.

Figure 3: Sample Doppler spectra and M-mode images from midpoint of M1 segment of the MCA. (**A**) Spectrum taken right after applying transducer to the temporal window, just in front of the ear. (**B**) Sample Doppler spectrum at same location and depth as (**A**). The only change is that the transducer has been angled upwards (superiorly) slightly. In both (**A**) and (**B**), depth = 50 mm, gain = 50, sample volume = 12 mm, power = 420 mW/cm^2, and filter = 100 Hz.

Figure 4: Spectral Doppler (top) and M-mode (bottom) image of bifurcation of the ICA into the MCA and ACA. Depth = 65 mm, gain = 50, sample volume = 12 mm, power = 420 mW/cm^2, and filter = 100 Hz.

Figure 5: Subject wearing custom fixation device.

Figure 6: Sample Doppler spectra and M-mode images from the MCA during different stages of the breath-hold maneuver. (**A**) Spectrum and M-mode at the beginning of breath-holding. Vertical yellow line in center denotes the start of breath-holding. (**B**) Spectrum and M-mode at the end of breath-holding. Vertical yellow line in center denotes the end of breath-holding when the subject inhales. (**C**) Spectrum and M-mode after the end of breath-holding, showing the

decrease in flow velocity that persists for approximately 30 s after breath-holding. In all spectra, depth = 56 mm, gain = 50, sample volume = 8 mm, power = 420 mW/cm^2, and filter = 100 Hz.

Figure 7: Spectrum and M-mode from the MCA throughout breath-holding. Depth = 56 mm, gain = 50, sample volume = 8 mm, power = 420 mW/cm^2, and filter = 100 Hz. Breath-holding begins at the left side of the screen and ends at the vertical yellow line.

Figure 8: Examples of bilateral spectra and M-mode images from the MCA. (**A**) Acceptable, but not optimal, spectrum and M-mode of the left MCA, with depth = 62 mm, gain = 69, sample volume = 12 mm, power = 420 mW/cm^2, and filter = 100 Hz. (**B**) Good spectrum and M-mode of right MCA, with depth = 62 mm, gain = 56, sample volume = 12 mm, power = 420 mW/cm^2, and filter = 100 Hz. (**C**) Good spectrum and M-mode of the left MCA. (**D**) Good spectrum and M-mode of the right MCA. For both (**C**) and (**D**), depth = 62 mm, gain = 56, sample volume = 12, power = 420 mW/cm^2, and filter = 100 Hz.

Table 1: MCA depths at various ages. Sources: a = Bode[25], b = Alexandrov et al.[10]

Table of Materials. Equipment used for the breath-holding experiment. Equipment listed is not meant as an endorsement; for each piece of equipment listed, many suitable alternatives exist.

DISCUSSION:

Critical steps in the protocol include 1) finding the MCA, 2) placing the headband, and 3) performing the breath-holding maneuver.

Modifications may be necessary depending on the subjects in the study. For example, subjects with Alzheimer's disease may have difficulty following instructions, necessitating the use of a capnograph to ensure compliance with breath-holding instructions[15]. Young children may have difficulty following instructions and may be shy of the experimenter; hence, experimental protocols may need to be simplified for such a population (see Lohmann et al.[2]). Certain settings on the TCD machine may also need to be changed depending on the population of interest. For example, when insonating infants, who have thin cranial bones, reduce the power as much as possible, especially if TCD monitoring will take place over a period lasting several hours[16].

Troubleshooting often centers around difficulties finding a good, stable TCD spectral signal. For example, for people older than 50 years of age, the temporal acoustic window becomes increasingly smaller as the age increases due to increased porosity of the bone of the cranium and tends to localize to the region just ahead of the ear (the "intertragal space")[12]. In such a population, finding a good MCA spectral signal on both sides of the head may sometimes be impossible, and very slight changes in transducer angle or position may cause the signal to be lost. Because a good-quality signal is essential for experiments that depend on the envelope waveform for analysis, every effort should be made to increase MCA spectral signal intensity and quality. For instance, the gain can be adjusted to optimize the signal, and the sample volume can be increased to get a stronger signal. As a last resort, power may be increased. Finally, it is

important to note that in approximately 10% of patients, the temporal acoustic window may be absent[11,17]. The temporal acoustic window can be readily found in infants and small children and is hardest to find in adults over the age of 50.

Limitations of fTCD include the acquisition of CBFV information at one spatial location[17] rather than a wide field of view, albeit with very high temporal resolution. Thus, fTCD is a complement to fMRI, which gives cerebral hemodynamic information (and hence neural activity) with a wide field of view at a low temporal resolution[18,19]. Indeed, fTCD has a temporal resolution comparable to that of fNIRS[20], with the important difference that fTCD measures hemodynamic changes at the level of the major cerebral arteries, whereas fNIRS measures changes in the cortex. Therefore, fTCD can fill in **significant** details about cerebral hemodynamic changes in response to neural activation, which no other neuroimaging modality is currently capable of measuring.

Potential applications of TCD include monitoring for cerebral embolus formation during cardiac surgery[16] and monitoring to detect the outcome of tissue plasminogen activator treatment for stroke[21]. Potential applications of fTCD include any research question involving the neural response to internal or external stimuli, such as studying the lateralized processing of language in the human brain[2–4], somatosensory "touch" stimulation[5], or lateralization of visual processing[6]. In addition, fTCD can be used to study physiological (with or without neural activity changes) responses to stimuli such as exercise[22] and breath-holding[13,15,23]. Finally, the low cost, portability, and simplicity of fTCD make imaging of large numbers of subjects practical, an advantage over fMRI and other neuroimaging modalities such as PET, e.g., when screening for preclinical Alzheimer's disease[15].

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DISCLOSURES:
The authors declare no conflicts of interest.

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Figure 1

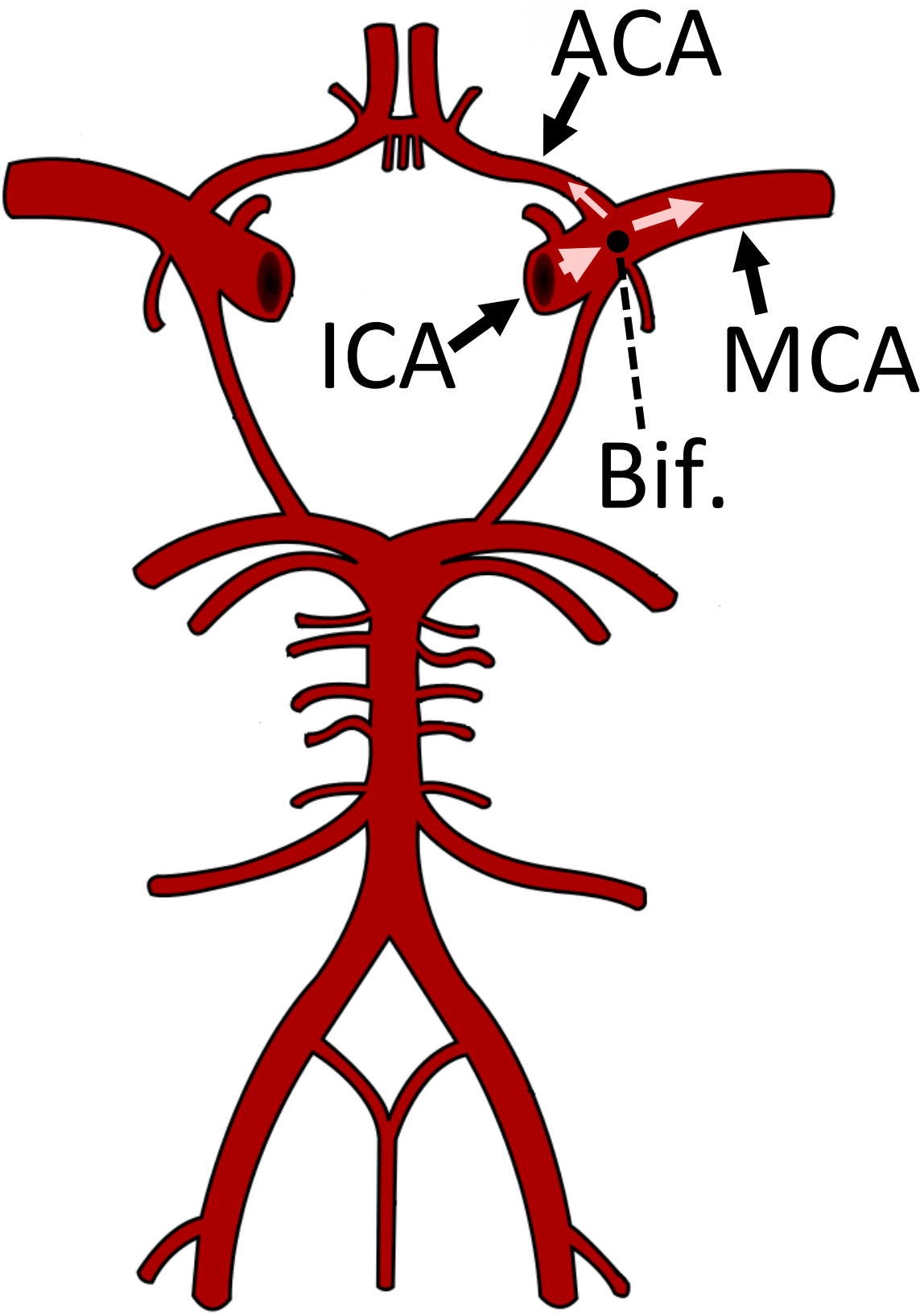

Figure 2

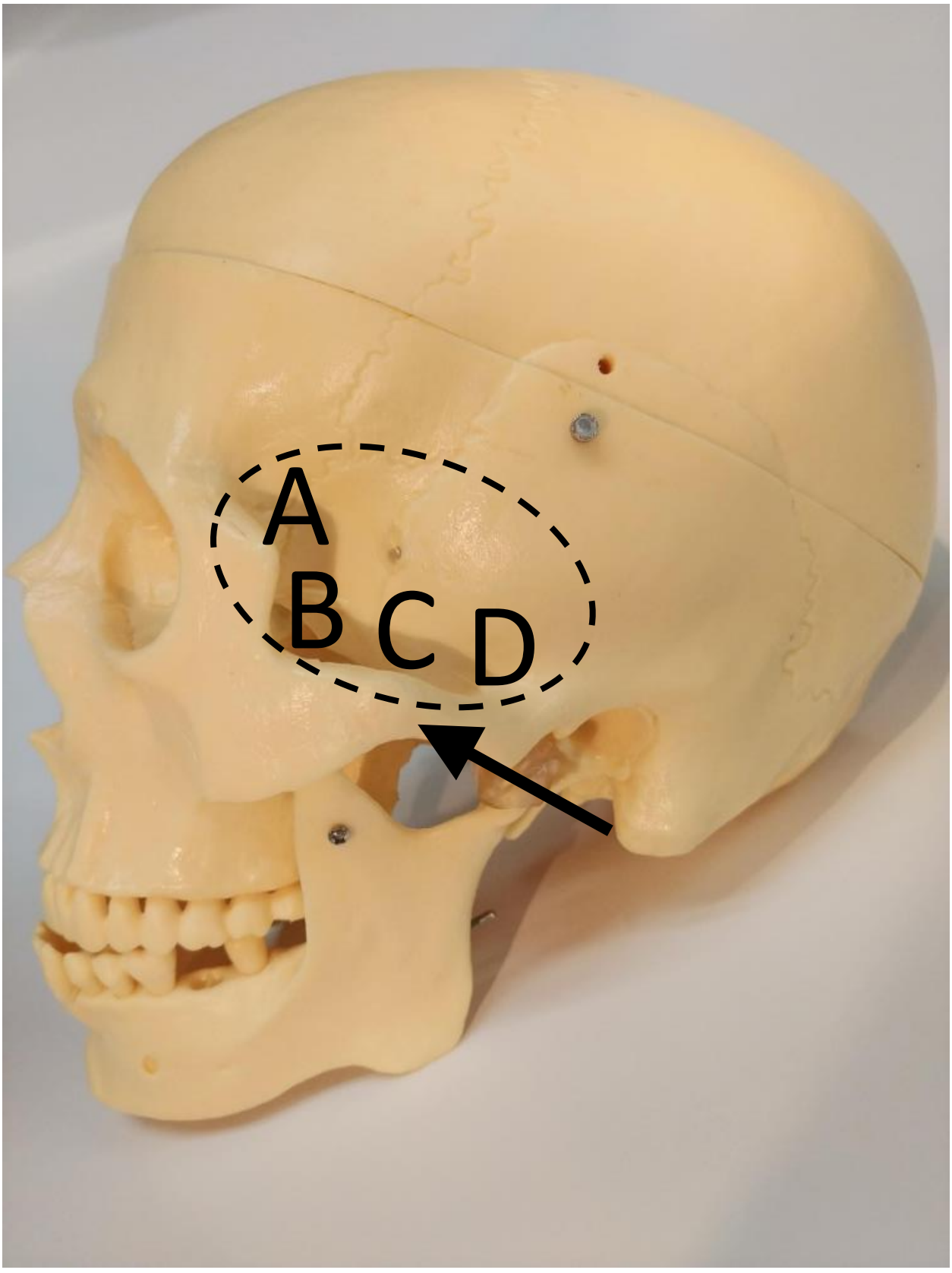

Figure 3

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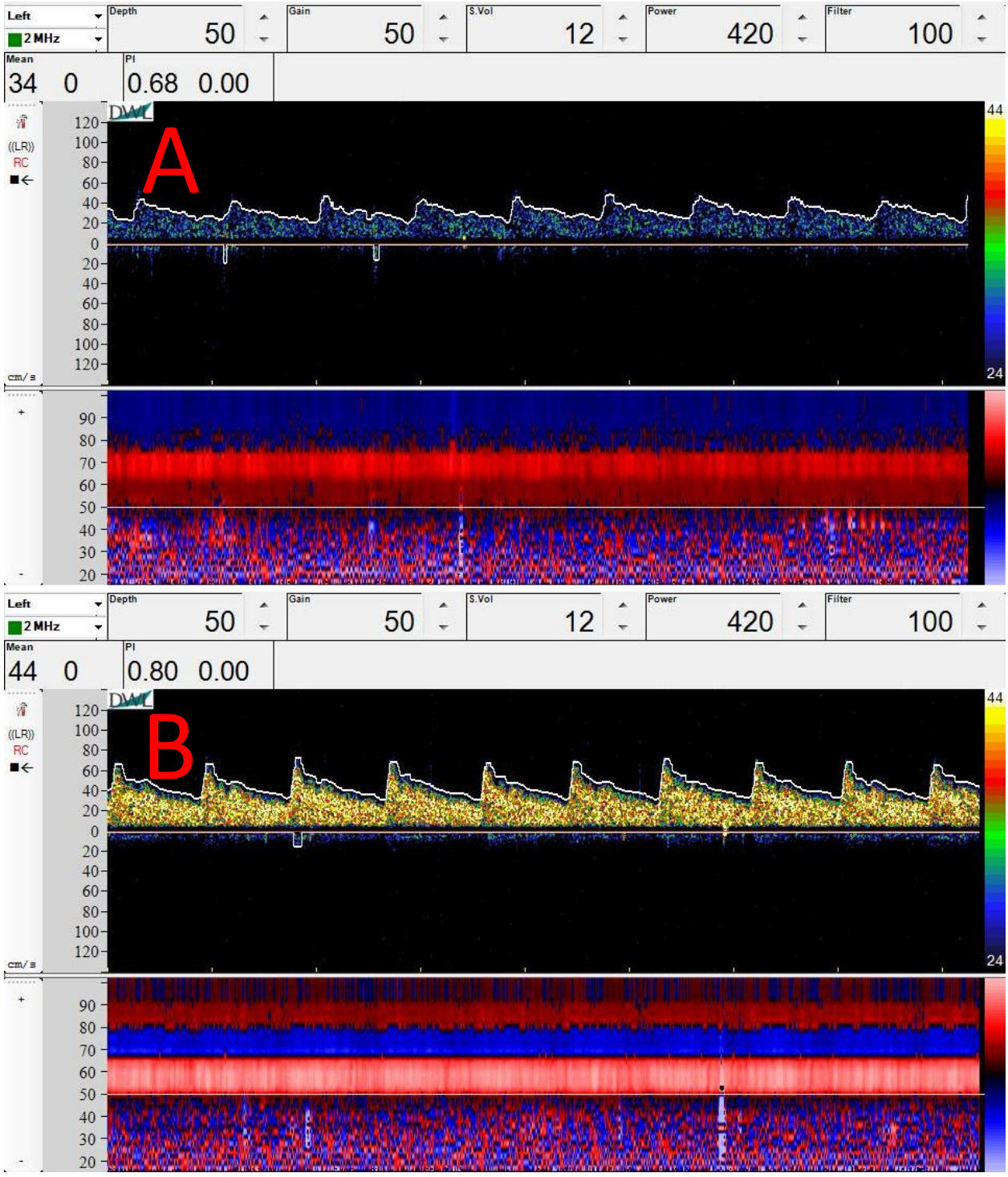

Figure 4

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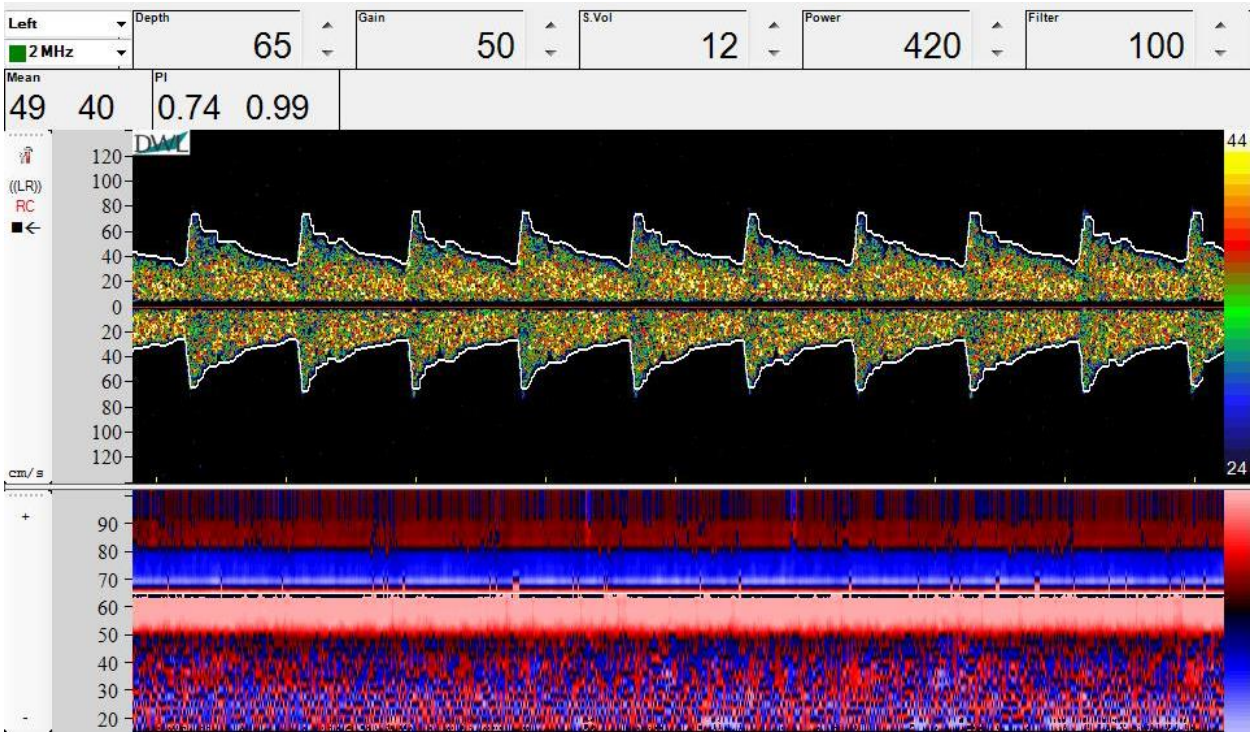

Figure 5

Figure 6

Click here to access/download;Figure;Figure_6.pdf

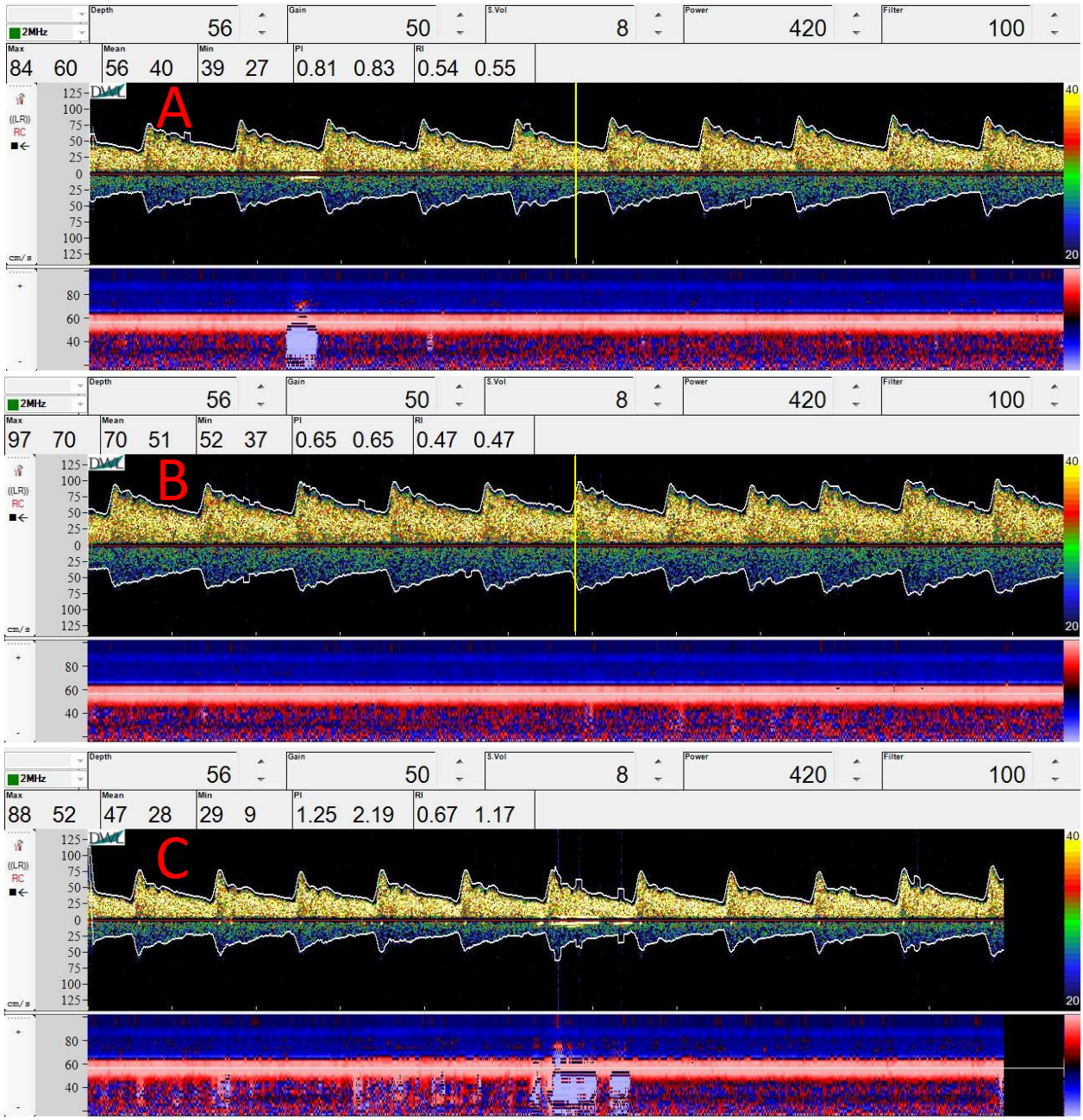

Figure 7

Click here to access/download;Figure;Figure_7.pdf

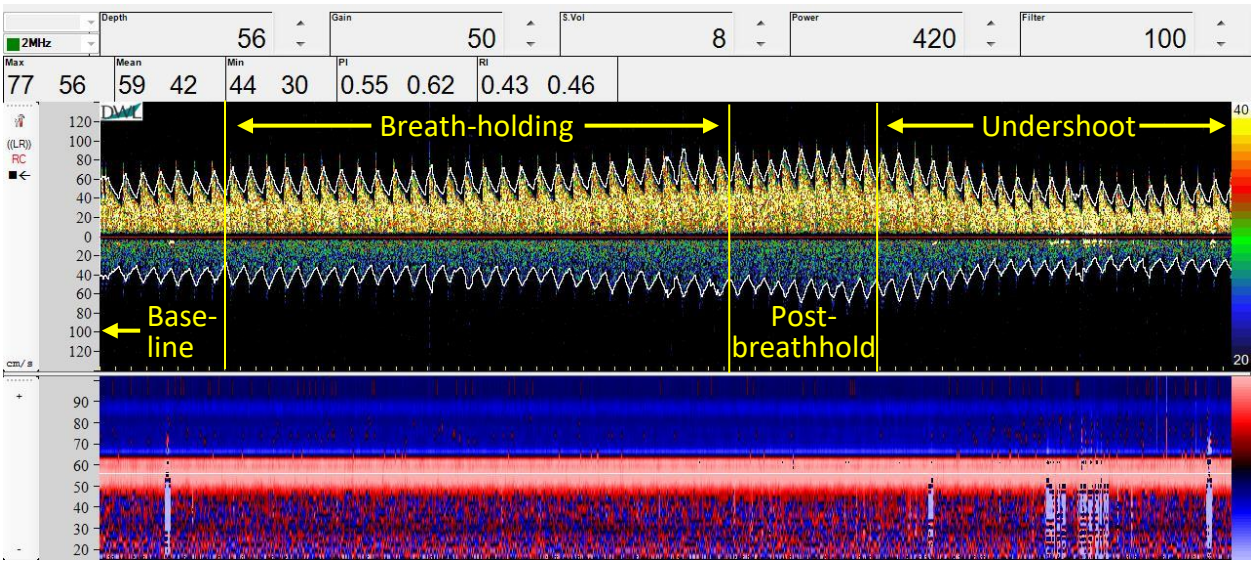

Figure 8

Click here to access/download;Figure;Figure_8.pdf

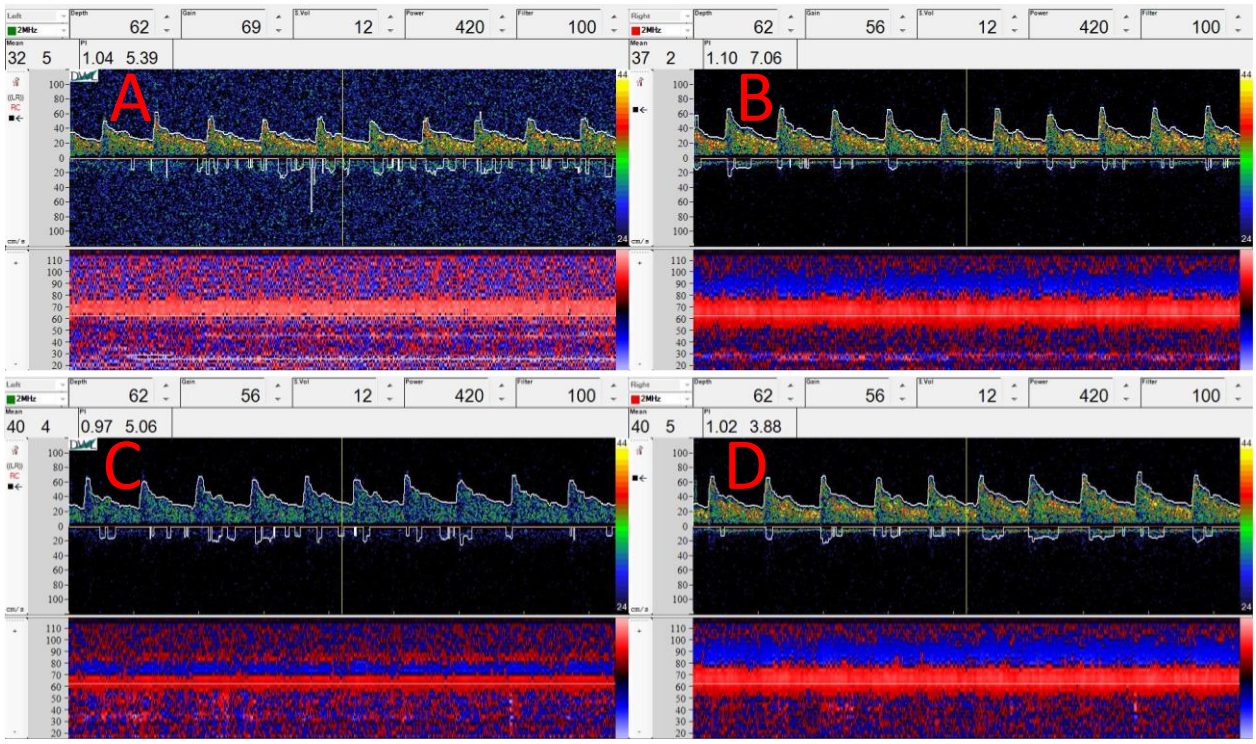

Table 1

Age	Middle cerebral artery depth (mm)
0–3 months[a]	25
3–12 months[a]	30
1–3 years[a]	35–45
3–6 years[a]	40–45
6–10 years[a]	45–50
10–18 years[a]	45–50
>18 years[b]	50

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_Bashford_alphabetical.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Aquasonic	Parker Laboratories, Inc., Fairfield, NJ, USA	01-50	Ultrasound Gel
Doppler Box X	DWL Compumedics Gmbh, Singen, Germany	Model "BoxX"	Transcranial Doppler with 2-MHz monitoring probes
Kimwipes	Kimberly-Clark Professional	34256	Delicate Task Wipers
Transeptic	Parker Laboratories, Inc., Fairfield, NJ, USA	09-25	Cleaning Spray

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you, we have done so.

2. 1.1.5: As you have specifically mentioned the depth to be set for adults, please specify depths for children/adolescents/infants.

Thank you for pointing out this omission. We have done this by adding Table 1. In point 1.1.5, we added the words, "Depth settings for children are given in **Table 1**."

3. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Only the titles and description for tables should appear after the Representative Results of the manuscript text.

We have done this. Please see the file "JoVE_Materials_Bashford_alphabetical.xlsx".

4. Figure 5: Please consider blurring or modifying the subject's face to de-identify the subject.

The subject's face in **Figure 5** has been blurred.

5. Please sort the Materials Table alphabetically by the name of the material.

The Materials Table has been sorted alphabetically. Please see the file "JoVE_Materials_Bashford_alphabetical.xlsx".

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes very detailed methodological step-by-step approach to so-called functional TCD examinations. Authors describing consequential stages how to set up and conduct testing while testing different functional tests, using as example testing cerebrovascular reactivity.

Major Concerns:

This manuscript does not represent any novelty, and represent composition of all well-known and already described many times methodological steps describing TCD monitoring method.

The manuscript is an invited methods article, and we agree that novelty was not part of the purpose of this educational paper.

Minor Concerns:

1. This manuscript needs substantial editing. Abbreviation must be placed after first mentioning of any parameter or label and not to repeat again.

Thank you for pointing this out. We have gone through the paper carefully to make sure that the abbreviation of a term is given the first time that the term is mentioned, and that thereafter only the abbreviation is used.

2. In Fig. 6A, 6B 6C authors indicating different CBFVs values, like 56 cm/sec for 6A or 70 cm/sec for fig. 6B; however, on the suggested figures no appropriate labels or numbers displayed. At the same time, TCD unit display from DWL Doppler Box-X shows all different parameters, like vessels labels, depth of insonation, values of CBFVs and few others variables. Therefore, all proposed figures must include full snapshot of TCD machine display.

We have added TCD machine parameters to **Figure 3**, **Figure 4**, **Figure 6**, **Figure 7, and Figure 8**.

3. Fig. 7 demonstrates three phases of breath-holding (breath-holding, post breath-hold and undershot); however, there is missing baseline which is absolutely necessary to demonstrate.

We agree that the baseline measurement would be helpful to see. We have adjusted **Figure 7** so that some of the baseline period is shown in the figure.

4. Number of figures must be decreased

Thank you for the suggestion, however, we believe that for the purpose of this instructional article, all of the figures are necessary.

5. Authors were also suggesting Table entitled "Name of Material/Equipment". Not clear why this list is offering because there are number of different TCD instruments that could be used to do breath-holding or any type of functional tests. Same comments about aquaponics ultrasound gel because for ultrasound tests there are number of ultrasound gel that can be used. It will be better if authors will listed all potential materials or equipment or not listed them at all and just making comment:

We regret the confusion we may have caused. For this Methods article, we listed the equipment that we used in our experiments, to allow replication of our methods. We recognize that similar equipment should achieve comparable results, and have added the following legend to the Table of Materials:

Table of Materials. Equipment used for the breath-holding experiment. Equipment listed is not meant as an endorsement; for each piece of equipment listed, many suitable alternatives exist.

Reviewer #2:

Manuscript Summary:

This is a nice TCD methodological paper.

It is well-written and will be a useful reference to trainees in this field.

I have no other comments.

Thanks!

We thank Reviewer 2 for their time and energy in reviewing the paper, and for the positive comments.

Reviewer #3:

Manuscript Summary:

The Methods paper presented techniques for using TCD to assess functional changes in brain blood flow. The basic steps for identifying and optimizing the signal from the middle cerebral artery was described, as well as placement of the fixation device and a breath-hold maneuver as one example of a functional use for the device. The descriptions are clear an concise.

Major Concerns:

There are no major concerns, except that although ultrasound is classically an imaging modality, Doppler ultrasound in this case does not rely on imaging per se to derive responses, changes, etc. Hence, the use of imaging can seem misleading in this application

We appreciate this comment and the chance to clarify the use of “image” in a neuroimaging context. We have revised the introduction to say: “Functional transcranial Doppler ultrasound (fTCD) is classified as a neuroimaging modality. In fTCD, “imaging” means dimensions of time and space, rather than the two dimensions of space which are more familiar in an “image.”

Minor Concerns:

There are only tow very minor concerns. It is noted for the breath hold that "The subject should not inhale deeply, as this would be a Valsalva maneuver14, which causes pressure to build up in the thorax." This is incorrect. The Valsalva maneuver requires the subject to substantially increase intrathoracic pressure against a held inspiration. A better reason for not inhaling deeply would be to avoid decreasing CO2 and reducing the likelihood of observing the increase in cerebral flow induced by the hypercapnia of a breath hold.

We appreciate the reviewer’s comment and suggestion, and have corrected the sentence to read, “The subject should not inhale deeply, as this would decrease carbon dioxide in the lungs and decrease the likelihood of observing the increase in CBFV due to cerebrovascular reactivity.”

To make the nature of the breath-holding maneuver absolutely clear to the reader, we have retained the sentence, “The subject should also avoid performing a Valsalva maneuver, in which intrathoracic pressure is substantially increased against a held inspiration[14].”

Also, in reference to this, Figure 7 should show the tracing pre breath hold to appreciate the consequent rise in flow.

Thank you. We have changed Figure 7 accordingly (see response to Reviewer 1, Question 3).