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Title: Repeated Cerebral Blood Flow Measurement with Laser Speckle Contrast Imaging Using an Acrylic Helmet in Murine Cerebral Malaria

Authors and Affiliations:

Melina Pedroso Merlone^{1,2}, Jhonata Willy Rocha Coelho³, Isabele Barbieri do Santos³, Fabiana Gomes da Conceição^{1,2}, Guilherme Salgado Sanches^{1,2}, Cláudio Tadeu Daniel-Ribeiro^{1,2}, Leonardo José de Moura Carvalho^{1,2}

¹Laboratory of Malaria Research, Instituto Oswaldo Cruz, IOC-Fiocruz

²National Institute of Science and Technology on Neuroimmunomodulation – INCT-NIM, IOC-Fiocruz

³Animal Experimentation Center, IOC-Fiocruz

Corresponding Authors:

Leonardo José de Moura Carvalho leojmc@ioc.fiocruz.br; ljmcarvalho@gmail.com

Email Addresses for All Authors:

Melina Pedroso Merlone	melinamerlone@gmail.com ,
Jhonata Willy Rocha Coelho	jhonata.coelho@ioc.fiocruz.br
Isabele Barbieri do Santos	belebarbieri@yahoo.com.br
Fabiana Gomes da Conceição	g.fabiana@gmail.com
Guilherme Salgado Sanches	guilhermessanches21@gmail.com
Claudio Tadeu Daniel-Ribeiro	malaria@fiocruz.br
Leonardo José de Moura Carvalho	leojmc@ioc.fiocruz.br ; ljmcarvalho@gmail.com

Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No.**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes.**

If **Yes**, please provide the **full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author willing to participate.

Melina Pedroso Merlone, PhD student; Leonardo José de Moura Carvalho, Senior Investigator (IOC-Fiocruz)

Current Protocol Length

Number of Steps: 24

Number of Shots: 57

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: *Answers are slightly edited in accordance with journal guidelines. Please note that the questions will not appear on screen. Please answer in **stand-alone full sentence with sufficient context***

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Melina Pedroso Merlone**: Our research focuses on understanding experimental cerebral malaria pathophysiology and adjuvant therapy effects through cerebral blood flow measurements.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Leonardo J. M. Carvalho**: Repeated cerebral blood flow measurements using LSCI are challenging; whereas this protocol is simple, minimally invasive, and reduces animal numbers.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Melina Pedroso Merlone**: This protocol allows measurement of cerebral blood flow from infection onset through cerebral malaria development, as well as the assessment of treatment effects.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Melina Pedroso Merlone**: The most important consideration is applying cyanoacrylate glue and keeping the mouse alone in the cage to prevent removal of the acrylic layer.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Leonardo J. M. Carvalho:** Future studies can measure cerebral blood flow in mice during disease and investigate the effects of treatments.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What value do you expect this video article to have for other researchers working in your field?

- 1.6. **Melina Pedroso Merlone, PhD student; Leonardo José de Moura Carvalho, Senior Investigator (IOC-Fiocruz)**: (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

How does the video format of your article help you to share your work with the broader scientific community?

- 1.7. **Melina Pedroso Merlone, PhD student; Leonardo José de Moura Carvalho, Senior Investigator (IOC-Fiocruz)**: (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

This research has been approved by the Ethics Committee on Animal Use at Instituto Oswaldo Cruz-Fiocruz

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Acrylic Helmet Implantation

Demonstrator: Melina Pedroso Merlone

- 2.1. To begin, obtain male and female 12 to 20-week-old mice [1-TXT]. Prepare a 1-milliliter syringe fitted with a 0.3-by-13-millimeter needle [2]. Inject the 45 microliters of anesthetic intramuscularly into the mouse [3-TXT].
 - 2.1.1. WIDE: Talent walking into the room with the mice in a cage. **TXT: Mouse strain: C57BL/6** **Videographer:** Please obtain CU shot as well for this shot.
 - 2.1.2. Shot of a 1 milliliter syringe fitted with a 0.3 × 13-millimeter needle.
 - 2.1.3. Talent administering the anesthetic injection intramuscularly into the mouse. **TXT: Anesthesia: Ketamine 75 mg/kg + Xylazine 7 mg/kg**
- 2.2. Prepare a 1-milliliter syringe fitted with a 0.3 by 13-millimeter needle [1]. Inject 200 microliters of analgesic diluted in water subcutaneously into the mouse [2-TXT].
 - 2.2.1. Talent preparing a 1 milliliter syringe fitted with a 0.3 × 13-millimeter needle.
 - 2.2.2. Talent administering the buprenorphine injection subcutaneously into the mouse. **TXT: Analgesia: Buprenorphine 1.0 mg/kg**
- 2.3. Check the depth of anesthesia by verifying the loss of the palpebral, tail, and pedal withdrawal reflex, and proceed only if the mouse does not respond [1].
 - 2.3.1. Talent checking the palpebral, tail, and pedal withdrawal reflex of the mouse and confirming the absence of response.
- 2.4. Place the mouse in a stereotaxic apparatus [1]. Carefully restrain the head with ear bars [2]. Hydrate the eyes with ophthalmic gel [3].
 - 2.4.1. Talent placing the mouse in the stereotaxic apparatus.
 - 2.4.2. Talent securing the mouse's head with ear bars.
 - 2.4.3. Talent applying ophthalmic gel to the eyes.
- 2.5. Using a scalpel blade, trimmer, or depilatory cream, remove the hair from the back of the neck up to the eyes [1]. Clean the skin with a swab containing 10% iodopovidone aseptic solution [2].
 - 2.5.1. Talent removing hair from the back of the neck up to the eyes using scalpel blade.

- 2.5.2. Talent cleaning the exposed skin with a swab containing 10% iodopovidone solution.
- 2.6. Using tweezers, hold the skin on the top of the head [1]. Using scissors, remove the skin to form an elliptical opening [2]. Retract the periosteal membrane with tweezers to expose the skull [3]. Clean the exposed skull surface with a swab soaked in 0.9 percent saline solution [4]. Dry the skull surface with gauze [5].
 - 2.6.1. Shot of the skin being held on the top of the head with tweezers.
 - 2.6.2. Shot of the skin being cut and removed to create an elliptical opening using scissors.
 - 2.6.3. Shot of periosteal membrane being retracted with tweezers to expose the skull.
 - 2.6.4. Shot of the exposed skull surface being cleaned with a saline-soaked swab.
 - 2.6.5. Shot of the skull surface being dried with gauze.
- 2.7. Fill a microcentrifuge tube with 500 microliters of dental uncolored powder [1]. Aspirate 500 microliters of liquid monomer [2]. Transfer the powder and liquid into a recipient [3]. Mix with a small plastic shovel until a homogeneous liquid is obtained [4]. Wait for the mixture to thicken slightly [5].
 - 2.7.1. Shot of the microcentrifuge tube being filled with dental uncolored powder polymer.
 - 2.7.2. Shot of the liquid monomer being aspirated.
 - 2.7.3. Shot of the powder and liquid being transferred into a recipient.
 - 2.7.4. Talent mixing the components with a small plastic shovel until the liquid is homogeneous.
 - 2.7.5. Shot of the acrylic resin mixture thickened.
- 2.8. Apply 3 to 4 small drops of veterinary cyanoacrylate-based glue on each side of the skull surface [1]. Using the small plastic shovel, spread the dental cement over the entire surface of the skull, leaving no openings [2]. Extend the dental cement slightly beyond the edge and above the skin to improve adhesion [3].
 - 2.8.1. Shot of veterinary cyanoacrylate-based glue being applied to each side of the skull surface.
 - 2.8.2. Shot of dental cement being spread over the skull surface with a small plastic shovel, leaving no openings.
 - 2.8.3. Shot of dental cement being extended beyond the skull edge and above the skin.
- 2.9. Using the small plastic shovel, model the acrylic layer to form a thin center measuring 0.1 to 0.2 millimeters. Create thicker edges for stability and protection [1]. Allow the resin to dry until rigid and no longer moldable to form the acrylic helmet [2].

- 2.9.1. Shot of the center of the acrylic layer being shaped to form a thin center using small plastic shovel
- 2.9.2. ~~Shot of the thicker acrylic edges being created.~~ **NOTE: Not filmed, VO merged**
- 2.9.3. Talent placing the preparation aside to dry and form the acrylic helmet.
- 2.10. Verify that the brain blood vessels are visible through the acrylic helmet [1]. Confirm that the acrylic helmet is not opaque and that no bubbles or white spots are present [2].
 - 2.10.1. Shot of brain blood vessels visible through the acrylic helmet.
 - 2.10.2. Shot of the transparent acrylic helmet showing the absence of bubbles and white spots in the acrylic helmet.
- 2.11. Prepare a 1-milliliter syringe fitted with a 0.3-by-13-millimeter needle [1]. Inject 100 microliters of diluted meloxicam subcutaneously [2-TXT].
 - 2.11.1. Talent preparing a 1 milliliter syringe fitted with a 0.3 × 13-millimeter needle.
 - 2.11.2. Talent administering the meloxicam injection subcutaneously into the mouse.
TXT: Meloxicam 1.0 mg/kg
- 2.12. Remove the mouse from the stereotaxic apparatus [1]. Return the mouse to an individual cage [2]. Cover the mouse with cotton and keep it under observation until full recovery from anesthesia [3].
 - 2.12.1. Talent removing the mouse from the stereotaxic apparatus.
 - 2.12.2. Talent placing the mouse into an individual cage.
 - 2.12.3. Talent covering the recovering mouse with cotton inside the cage.
- 2.13. The following day, administer another dose of buprenorphine subcutaneously as previously demonstrated [1].
 - 2.13.1. Talent administering the buprenorphine injection subcutaneously into the mouse.

3. Laser Speckle Contrast Imaging and Cerebral Blood Flow Analysis

- 3.1. Perform laser speckle contrast imaging or LSCI (*L-S-C-I*) measurements 2 or 7 days after acrylic helmet implantation in mice previously infected with *Plasmodium berghei* ANKA (*Anca*) and treated with antimalarial drugs [1].
 - 3.1.1. Shot of a mouse with an acrylic helmet prepared for imaging.
- 3.2. Turn on the LSCI equipment at least 10 minutes before use to allow the laser time to warm up [1].

- 3.2.1. Talent turning on the LSCI equipment.
- 3.3. Place a heating pad set to 36 degrees Celsius under the LSCI system [1].
 - 3.3.1. Talent positioning the heating pad beneath the LSCI system.
- 3.4. Set the LSCI program by selecting **File** and choosing **New Recording** [1]. Enter the details such as the project name, site, subject, and the operator's name [2]. Set the measurement area width to 1.5 centimeters and height to 1.7 centimeters, and select normal point density [3]. Then, set the frame rate to 12 images per second, the duration until stopped, enable record with averaging, and choose 5 frames per second [4]. Select one color photo at the beginning, enable **live calculations** in performance, and disable **manual distance** [5].
 - 3.4.1. SCREEN: 71596_PIMSoft_ALL 00:03-00:09.
Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up.
 - 3.4.2. SCREEN: 71596_PIMSoft_ALL 00:22-:00:25, 00:55-00:57 and 01:04--1:09.
 - 3.4.3. SCREEN: 71596_PIMSoft_ALL 1:10-1:18 .
 - 3.4.4. SCREEN: 71596_PIMSoft_ALL 1:18-1:20 *Video editor: Highlight the frame rate and duration boxes as the timestamps are very short*
 - 3.4.5. SCREEN: 71596_PIMSoft_ALL 1:20-1:23 *Video editor: Highlight live calculations and Manual Distance.*
- 3.5. ~~After anesthetizing the mouse, place it on the heating pad [1-TXT].~~ Position the nose cone over the anesthetized animal's muzzle [2-TXT].
 - 3.5.1. ~~Talent placing the anesthetized mouse on the heating pad. TXT:~~
 - 3.5.2. Talent positioning the anesthetic output at the mouse's muzzle. **TXT:**
Anesthesia:
Induction: Isoflurane 5%; Gas flow: 0.80 - 0.85 L/min O₂ + Isoflurane
Maintenance: Isoflurane 1 - 1.8%; Gas flow: 0.70 - 0.85 L/min O₂ + Isoflurane
- 3.6. Once the acrylic helmet is cleaned with wet cotton, dry it with gauze [1]. Position the mouse's head under the red laser light [2]. Make small marks along the edge of the acrylic helmet with a permanent marker to guide the positioning of the region of interest or ROI (**R-O-I**) during analysis [3].
 - 3.6.1. Talent drying the acrylic helmet with gauze.
 - 3.6.2. Talent positioning the mouse's head under the red laser light.
 - 3.6.3. Talent marking the edge of the acrylic helmet with a permanent marker.
- 3.7. Verify that the acrylic helmet area appears on the computer screen [1]. ~~Adjust the equipment height until the distance between the measured area and the laser is 10~~

centimeters **[2]**.

3.7.1. SCREEN: 71596_PIMSOFT_ALL 1:23-1:26.

~~3.7.2. Talent adjusting the equipment height while monitoring the distance value.~~

3.8. Draw an ellipse over the ROI for measurement by selecting the **Ellipse** tool **[1]**. Then, draw a small circle in the corner without blood flow by selecting the **Circle** tool **[2]**. Adjust the equipment height until the distance between the measured area and the laser is 10 centimeters **[3]**. Position the elliptical ROI over the skull and stretch it to cover the thin acrylic layer, excluding the thicker edges **[4]**.

3.8.1. SCREEN: 71596_PIMSOFT_ALL 1:29-1:34

3.8.2. SCREEN: 71596_PIMSOFT_ALL 1:39-1:43.

3.8.3. SCREEN: 71596_PIMSOFT_ALL 2:05-2:17

3.8.4. SCREEN: 71596_PIMSOFT_ALL . 2:17-2:20 and 2:50-2:57.

3.9. Click on the **Play** icon and record for 5 minutes **[1]**. Once done, stop the recording by selecting the **Stop** icon and close the recording to save it automatically **[2]**. Return the mouse to its cage and monitor it until it regains consciousness. Repeat the imaging measurements after 4, 5, and 6 days **[3]**.

3.9.1. SCREEN: 71596_PIMSOFT_ALL 2:57-3:00 and 03:15-03:19

3.9.2. SCREEN: 71596_PIMSOFT_ALL 03:25-3:35.

3.9.3. Talent returning the mouse to its cage.

~~3.10. Repeat the imaging measurements after 4, 5, and 6 days **[1]**.~~

~~3.10.1. Shot representing repeated LSCI sessions on subsequent days.~~ **NOTE: Not filmed, VO moved to the previous step**

3.11. After euthanizing the mouse, analyze the ROI measurements **[1-TXT]**.

3.11.1. SCREEN: 71596_PIMSoft_3.11.1 00:12-00:22. **TXT: Euthanasia: Ketamine 300 mg/kg + Xylazine 30 mg/kg, i.p.**

Results

4. Results

- 4.1. The dental acrylic formed a thin, transparent layer that remained stable without becoming opaque over the 15-day follow-up period [1].
 - 4.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the pink transparent area on the skull shown across the images labeled 'D1', 'D5', 'D6', 'D7', and 'D13'.*
- 4.2. The thin acrylic layer and the skull itself were permeable to laser penetration, allowing measurements of blood flow underneath [1] and allowing blood flow imaging beneath the skull [2].
 - 4.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the colored blood-flow map inside the white outlined region in all the images.*
 - 4.2.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the colored blood-flow image inside the white outlined region in all the images.*
- 4.3. No significant differences in cerebral blood flow were observed between measurements performed on day 1 [1], day 5 [2], day 6 [3], and day 7 [4].
 - 4.3.1. LAB MEDIA: Figure 3A, B. *Video editor: Highlight the data points above the label 'D1'.*
 - 4.3.2. LAB MEDIA: Figure 3A, B. *Video editor: Highlight the data points above the label 'D5'.*
 - 4.3.3. LAB MEDIA: Figure 3A, B. *Video editor: Highlight the data points above the label 'D6'.*
 - 4.3.4. LAB MEDIA: Figure 3A, B. *Video editor: Highlight the data points above the label 'D7'.*
- 4.4. A significant decrease in cerebral blood flow was detected on day 15 [1] compared to day 7 [2].
 - 4.4.1. LAB MEDIA: Figure 3A,B. *Video editor: Highlight the data points above the label 'D15'.*
 - 4.4.2. LAB MEDIA: Figure 3A,B. *Video editor: Highlight the data points above the label 'D7'.*
- 4.5. The laser signal was not significantly affected between the measurements before [1] and after implantation of the thin acrylic layer [2].
 - 4.5.1. LAB MEDIA: Figure 4. *Video editor: Highlight the data points above the label*

'Before' in panel B.

4.5.2. LAB MEDIA: Figure 4. *Video editor: Highlight the data points above the label 'After' in panel B.*

Anesthesia

Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthesia>

IPA: /ˌæn.əsˈθiː.ʒə/, /ˌæn.əsˈθiː.zi.ə/

Phonetic Spelling: an·uhs·THEE·zhuh; an·uhs·THEE·zee·uh

Ketamine

Pronunciation link: <https://www.merriam-webster.com/dictionary/ketamine>

IPA: /ˈkiːtəˌmiːn/

Phonetic Spelling: KEE·tuh·meen

Xylazine

Pronunciation link: <https://www.merriam-webster.com/dictionary/xylazine>

IPA: /ˈzaɪləˌziːn/

Phonetic Spelling: ZYE·luh·zeen

Analgesic

Pronunciation link: <https://www.merriam-webster.com/dictionary/analgesic>

IPA: /ˌæn.əlˈdʒiːzɪk/

Phonetic Spelling: an·uhl·JEE·zik

Buprenorphine

Pronunciation link: <https://www.merriam-webster.com/dictionary/buprenorphine>

IPA: /ˌbjuːprəˈnɔːrfiːn/

Phonetic Spelling: byoo·pruh·NOR·feen

Palpebral

Pronunciation link: <https://www.merriam-webster.com/dictionary/palpebral>

IPA: /ˈpælpəbrəl/

Phonetic Spelling: PAL·puh·bruhl

Stereotaxic

Pronunciation link: <https://www.merriam-webster.com/dictionary/stereotaxic>

IPA: /ˌsteri.oʊˈtæksɪk/

Phonetic Spelling: STEHR·ee·oh·TAK·sik

Ophthalmic

Pronunciation link: <https://www.merriam-webster.com/dictionary/ophthalmic>

IPA: /ɑːfˈθælmɪk/

Phonetic Spelling: ahf·THAL·mik

Depilatory

Pronunciation link: <https://www.merriam-webster.com/dictionary/depilatory>

IPA: /dɪˈpɪləˌtɔːri/

Phonetic Spelling: dih·PIL·uh·tor·ee

Iodopovidone

Pronunciation link: No confirmed link found

IPA: No verified American IPA found

Phonetic Spelling: eye·oh·doh·POV·ih·dohn

Periosteal

Pronunciation link: <https://www.merriam-webster.com/dictionary/periosteum>

IPA: /ˌperiˈɑːstiəl/

Phonetic Spelling: pehr·ee·OSS·tee·uhl

Microcentrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /ˌmaɪkroʊˈsentrɪˌfjuːdʒ/

Phonetic Spelling: MY·kroh·SEN·truh·fyooj

Monomer

Pronunciation link: <https://www.merriam-webster.com/dictionary/monomer>

IPA: /ˈmɑːnəmər/

Phonetic Spelling: MON·uh·mer

Cyanoacrylate

Pronunciation link: <https://www.merriam-webster.com/dictionary/cyanoacrylate>

IPA: /saɪˌænoʊˈækɹəˌleɪt/

Phonetic Spelling: sigh·AN·oh·AK·ruh·layt

Acrylic

Pronunciation link: <https://www.merriam-webster.com/dictionary/acrylic>

IPA: /əˈkrɪlɪk/

Phonetic Spelling: uh·KRIL·ik

Meloxicam

Pronunciation link: <https://www.merriam-webster.com/dictionary/meloxicam>

IPA: /məˈlɔːksɪˌkæm/

Phonetic Spelling: muh·LOK·sih·kam

Speckle

Pronunciation link: <https://www.merriam-webster.com/dictionary/speckle>

IPA: /'spɛkəl/
Phonetic Spelling: SPEK·uhl

Cerebral
Pronunciation link: <https://www.merriam-webster.com/dictionary/cerebral>
IPA: /sə'ri:brəl/
Phonetic Spelling: suh·REE·bruhl

Isoflurane
Pronunciation link: <https://www.merriam-webster.com/dictionary/isoflurane>
IPA: /,aɪsoʊ'florem/
Phonetic Spelling: EYE·soh·FLUR·ayn

Muzzle
Pronunciation link: <https://www.merriam-webster.com/dictionary/muzzle>
IPA: /'mʌzəl/
Phonetic Spelling: MUZ·uhl

Ellipse
Pronunciation link: <https://www.merriam-webster.com/dictionary/ellipse>
IPA: /ɪ'lips/
Phonetic Spelling: ih·LIPS

Plasmodium
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmodium>
IPA: /pləz'moʊdiəm/
Phonetic Spelling: plaz·MOH·dee·uhm

Berghei
Pronunciation link: No confirmed link found
IPA: No verified American IPA found
Phonetic Spelling: BERG·high

ANKA
Pronunciation link: No confirmed link found
IPA: No verified American IPA found
Phonetic Spelling: ANK·uh

Antimalarial
Pronunciation link: <https://www.merriam-webster.com/dictionary/antimalarial>
IPA: /,æn.ti.mə'ler.i.əl/
Phonetic Spelling: an·tee·muh·LAIR·ee·uhl

Euthanizing
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>

IPA: /'ju:θəˌnaɪz/

Phonetic Spelling: YOO·thuh·nyz

Permeable

Pronunciation link: <https://www.merriam-webster.com/dictionary/permeable>

IPA: /'pɜːmiəbəl/

Phonetic Spelling: PER·mee·uh·buhl

Opaque

Pronunciation link: <https://www.merriam-webster.com/dictionary/opaque>

IPA: /oʊ'peɪk/

Phonetic Spelling: oh·PAYK