

Submission ID #: 71542

Scriptwriter Name: Pallavi Sharma

Project Page Link: <https://review.jove.com/account/file-uploader?src=21820358>

Title: X-Band Electron Paramagnetic Resonance for Monitoring of Melanin-Associated Radical Changes in Zebrafish Melanoma Xenografts In Vivo

Authors and Affiliations:

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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**
- 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**

Current Protocol Length

Number of Steps: 22

Number of Shots: 39

Interviews

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

INTRODUCTION:

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

- 1.1. **Katerina Makarova**: We developed a method to monitor melanin radicals in living zebrafish embryos using X-band electron paramagnetic resonance spectroscopy.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 2.7.1.*

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

- 1.2. **Katerina Makarova**: Current approaches cannot directly detect melanin radicals in living embryos. This protocol enables label-free monitoring using electron paramagnetic resonance.
 - 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. **Katerina Makarova**: Using this protocol, researchers can quantify melanin-associated radicals and assess their changes during melanogenesis, tumor progression, and drug treatment.
 - 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. **Katerina Makarova:** The key challenge while performing this protocol is consistent embryo loading and normalization, because sample position and embryo number strongly affect signal intensity.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 2.4.1.*

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

- 1.5. **Katerina Makarova:** Future studies can use this method to evaluate novel anti-melanoma therapies and investigate mechanisms regulating melanin-associated redox biology in melanoma..

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.

NOTE: Shots 1.6.1 and 1.7.1 recorded twice - once according to the instructions, the second time the talent speaks directly to the camera,

What problem in your field of research does your JoVE article address?

- 1.6. **Katerina Makarova, Senior Researcher, Medical University of Warsaw:** (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.

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

- 1.7. **Katerina Makarova, Senior Researcher, Medical University of Warsaw:** (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.

Protocol

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

2. Melanin Radical Detection by Electron Paramagnetic Resonance Spectroscopy

Demonstrator: Katerina Makarova

- 2.1. To begin, turn on the X-band electron paramagnetic resonance spectrometer and allow the system to stabilize according to the manufacturer's instructions [1].
 - 2.1.1. WIDE: Talent turning on the X-band electron paramagnetic resonance spectrometer. ***Videographer:** Please obtain CU shot as well for this shot.*
NOTE: 2.1.1.A extra shot added as a close up to be placed right after 2.1.1
- 2.2. Obtain zebra fish larvae transplanted with B16F10 (*B-Sixteen-F-Ten*) melanoma cells [1]. Transfer 20 to 30 zebrafish larvae at 3 to 5 days post-fertilization to a 1.5-milliliter microcentrifuge tube [2]. Fill the flat capillary for zebrafish larvae with 100 microliters of E3 medium [3].
 - 2.2.1. Shot of the container containing zebra fish larvae. **NOTE: Numbering is switched, use 2.2.2 for 2.2.1 and 2.2.1 for 2.2.2**
 - 2.2.2. Talent transferring zebrafish larvae to a 1.5-milliliter microcentrifuge tube.
 - 2.2.3. Talent filling the flat capillary with E3 medium. **NOTE: extra shot 2.2.3.1 to be put after 2.2.3**
- 2.3. Seal one end of the capillary with a plastic cap and ensure that the liquid column remains confined to the flat chamber [1].
 - 2.3.1. Talent sealing one end of the capillary with a plastic cap. **NOTE: shot 2.3.1 is recorded at the end of 2.2.3**
- 2.4. Transfer several larvae together with a small volume of E3 medium into a 100-microliter hematocrit capillary [1]. Remove excess liquid from the outer tubular section of the capillary while maintaining continuous liquid contact within the flat chamber [2].
 - 2.4.1. Talent transferring several larvae with E3 medium into a 100-microliter hematocrit capillary. **NOTE: extra shot 2.4.1.A to be put before 2.4.2**
 - 2.4.2. Talent removing excess liquid from the outer tubular section of the capillary.
- 2.5. Repeat the previous loading and excess liquid removal process until 20 to 30 larvae are loaded into the flat capillary [1].
 - 2.5.1. Shot of the capillary with 20 to 30 larvae.
- 2.6. Then, remove residual liquid from the tubular sections of the capillary [1].

- 2.6.1. Talent removing residual liquid from the tubular sections of the capillary.
- 2.7. Insert the capillary into the electron paramagnetic resonance spectrometer [1] and position the sample at the center of the resonator [2].
 - 2.7.1. Talent inserting the capillary into the electron paramagnetic resonance spectrometer.
 - 2.7.2. Talent positioning the sample at the center of the resonator.
- 2.8. Set the acquisition parameters in the spectrometer software by entering a microwave frequency of 9.6 gigahertz, a center field of 335 millitesla, and a sweep width of 10 millitesla [1]. Apply a modulation amplitude of 500 microtesla and a microwave power of 1.6 milliwatts [2]. Next, set the number of scans to 32, the sweep time to 11 seconds, and the time constant to 0 [3]. Click Set parameters to transfer parameters to spectrometer, then click Tune [4].
 - 2.8.1. SCREEN: : 71542_screenshot_1.avi: 00:07-00:20
 - 2.8.2. SCREEN: 71542_screenshot_2.avi: 00:02-00:15
 - 2.8.3. SCREEN: 71542_screenshot_3.avi: 00:02-00:12
 - 2.8.4. SCREEN: 71542_screenshot_4.avi: 00:02-00:12
- 2.9. Acquire the electron paramagnetic resonance spectrum [1].
 - 2.9.1. SCREEN: 71542_screenshot_5.avi: 00:02-00:04, 00:18-00:20
- 2.10. Then, remove the capillary from the resonator [1] and release the larvae into fresh E3 medium in a Petri dish [2].
 - 2.10.1. Talent removing the capillary from the resonator.
 - 2.10.2. Talent releasing the larvae into fresh E3 medium in a Petri dish.

3. Melanin Radical Detection in Frozen Zebrafish Larvae

Demonstrator: Katerina Makarova

- 3.1. Thaw a frozen sample containing 10 euthanized zebrafish larvae at room temperature for 5 to 10 minutes [1]. Add 100 microliters of E3 medium to the thawed sample [2].
 - 3.1.1. Talent handling the frozen sample and placing it at room temperature for thawing.
 - 3.1.2. Talent adding E3 medium to the thawed sample.
- 3.2. Transfer the larvae together with the medium into a 100-microliter hematocrit capillary [1]. Remove excess liquid from the capillary until the sample occupies only the active resonator volume of about 1 centimeter in length [1]. Seal the capillary with a sealing compound [1].

- 3.2.1. Talent transferring the larvae and medium into a 100-microliter hematocrit capillary.
- 3.2.2. Talent removing excess liquid from the capillary.
- 3.2.3. Talent sealing the capillary with a sealing compound.
- 3.3. Insert the capillary into the electron paramagnetic resonance spectrometer [1] and position the sample at the center of the resonator [2].
 - 3.3.1. Talent inserting the capillary into the electron paramagnetic resonance spectrometer.
 - 3.3.2. Talent positioning the sample at the center of the resonator.
- 3.4. Acquire the spectrum using the same settings used previously and save the electron paramagnetic resonance spectrum [1].
 - 3.4.1. SCREEN: 71542_screenshot_5.avi: 00:32-00:40

4. Extracting Electron Paramagnetic Resonance Data and Post-processing of Spectra

Demonstrator: Katerina Makarova

- 4.1. Open the recorded electron paramagnetic resonance spectrum in the multiharmonic analysis software [1]. Enable dual-channel signal processing by selecting **Use two channels** [2].
 - 4.1.1. SCREEN: 71542_screenshot_6.avi
 - 4.1.2. SCREEN: 71542_screenshot_7.avi
- 4.2. Set the number of processed harmonics to 30 [1]. Adjust the **Microwave phase/deg** (*Microwave-Phase or Deg*) parameter until symmetrical absorption and dispersion components are obtained [2].
 - 4.2.1. SCREEN: 71542_screenshot_8.avi
 - 4.2.2. SCREEN: : 71542_screenshot_9.avi
- 4.3. Then, adjust the **Modulation phase/deg** parameter until Channel 1 shows a pure absorption signal [1]. ~~Then, continue adjusting the parameter until Channel 2 displays a pure dispersion signal. [2].~~ **NOTE: The VO is edited for the deleted shot**
 - 4.3.1. SCREEN: 71542_screenshot_10.avi
 - ~~4.3.2. SCREEN: Display a pure dispersion signal in Channel 2 after adjustment of the Modulation phase/deg parameter. **NOTE: Not filmed**~~
- 4.4. Modify the **Modulation amplitude/G** (*Modulation-Amplitude-or-G*) parameter until the narrowest undistorted spectral line is obtained [1].
 - 4.4.1. SCREEN: 71542_screenshot_12.avi

4.5. Then, set the **Low pass/Hz** (*Low pass-Or-Hertz*) parameter to reduce high-frequency noise while preserving the spectral line shape [1].

4.5.1. SCREEN: : 71542_screenshot_13.avi

4.6. Select the **Noise suppression** tab and click **Enable noise suppression** [1]. Adjust the **Smoothing factor** and **Narrow line refinement** parameters until the signal-to-noise ratio improves without visible distortion of the spectral line [2].

4.6.1. SCREEN: 71542_screenshot_14.avi

4.6.2. SCREEN: 71542_screenshot_15.avi:

4.7. Next, go to the **Artifacts** tab. While observing the EPR spectrum, enable **Outlier correction** and **Bandwidth correction** one at a time, continuously monitoring both the noise level and the reconstructed EPR line shape [1].

4.7.1. SCREEN: 71542_screenshot_16.avi

4.8. Save the spectra in a format used by the data analysis software for further analysis [1]. Import the processed data into the analysis software and perform the double integration procedure [2].

4.8.1. SCREEN: 71542_screenshot_17.avi: 00:00-00:14

4.8.2. SCREEN: 71542_screenshot_18.avi: 00:19-00:23, 00:30-00:34, 00:51-00:53

Results

5. Results

5.1. Wild-type embryos exhibited a characteristic single-line electron paramagnetic resonance signal [1], whereas albino embryos showed no detectable electron paramagnetic resonance signal in the same spectral region at 5 days post-fertilization [2].

5.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the black curve labeled “WT 5dpf”*
LAB MEDIA: Figure 2. *Video editor: Highlight the gray curve labeled “Albino 5dpf”*

5.2. Analysis of wild-type embryos from 2 to 5 days post-fertilization revealed a significant increase in the double-integrated electron paramagnetic resonance signal, normalized per embryo [1].

5.2.1. LAB MEDIA: Figure 3A. *Video editor: sequentially highlight the bars from left to right.*

5.3. Raw electron paramagnetic resonance spectra from samples with 7 and 10 embryos, taken at 5 days post-fertilization, exhibited differences in signal amplitude that were proportional to the sample size [1].

5.3.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the 2 curves labeled “7 fish” and “10 fish” and focus on the difference in peak height.*

5.4. After normalization to the number of embryos per sample, the spectra from samples containing 7 and 10 embryos overlapped almost completely [1].

5.4.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the 2 overlapping curves labeled “normalized 10 fish” and “normalized 7 fish”.*

5.5. Electron paramagnetic resonance spectra obtained with a custom flat capillary and a standard commercially available capillary displayed highly comparable line shapes and resonance positions. [1].

5.5.1. LAB MEDIA: Figure 4A.

5.6. No statistically significant differences were observed between fresh and frozen samples across 6 independent biological replicates [1].

5.6.1. LAB MEDIA: Figure 4B.

5.7. Non-injected albino embryos showed no detectable electron paramagnetic resonance signals in the melanin-specific region [1], nor were any signals found in albino embryos injected with B16F10 cells [2].

5.7.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the curve labeled “Uninjected*

albino 4dpf".

- 5.7.2. LAB MEDIA: Figure 5A. *Video editor: Highlight the curve labeled "Injected B16F10 albino 4dpf"*.
- 5.8. Successful cell engraftment was confirmed by bright microscopy in albino embryos injected with B16F10 cells [1].
 - 5.8.1. LAB MEDIA: Figure 5B. *Video editor: Zoom in on the lower image labeled "Implanted B16F1 cells" and highlight the area indicated by the arrow.*
- 5.9. Injection into circulation provided consistent electron paramagnetic resonance signals linked to melanoma cells [1], while injection into the yolk sac yielded weak or inconsistent signals [2].
 - 5.9.1. LAB MEDIA: Figure 6. *Video editor: Highlight the solid gray curve labeled "B16F10 blood injected"*.
 - 5.9.2. LAB MEDIA: Figure 6. *Video editor: Highlight the dotted curve labeled "B16F10 yolk injected"*.
- 5.10. Embryos bearing B16F10 xenografts exhibited a modified electron paramagnetic resonance signal compared with wild-type embryos at corresponding developmental time points [1].
 - 5.10.1. LAB MEDIA: Figure 7. *Video editor: Compare the light bars labeled "B16F10" with the dark bars labeled "Control" at each developmental time point.*

1. Electron paramagnetic resonance
Pronunciation link: <https://www.merriam-webster.com/dictionary/electron%20paramagnetic%20resonance>
IPA: /ɪˈlek.trɑːn ˌper.ə.mægˈnet.ɪk ˈrez.ə.nəns/
Phonetic Spelling: ihˌlekˌtron pairˌuhˌmagˌnetˌik rezˌuhˌnuhns
2. Spectrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrometer>
IPA: /spekˈtrɑː.mə.t̬ər/
Phonetic Spelling: spekˌtrahˌmuhˌter
3. Zebrafish
Pronunciation link: <https://www.merriam-webster.com/dictionary/zebrafish>
IPA: /ˈziː.brəˌfɪʃ/ ; /ˈzeb.rəˌfɪʃ/
Phonetic Spelling: zeeˌbruhˌfish; zebˌruhˌfish
4. B16F10
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: beeˌsixˌteenˌeffˌten
5. Melanoma
Pronunciation link: <https://www.merriam-webster.com/dictionary/melanoma>
IPA: /ˌmel.əˈnoʊ.mə/
Phonetic Spelling: melˌuhˌnohˌmuh
6. Microcentrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>
IPA: /ˌmaɪ.kroʊˈsen.trəˌfjuːdʒ/
Phonetic Spelling: myˌkrohˌsenˌtruhˌfyooj
7. Hematocrit
Pronunciation link: <https://www.merriam-webster.com/dictionary/hematocrit>
IPA: /hɪˈmæt̬.ə.krɪt/
Phonetic Spelling: heeˌmatˌuhˌkrit
8. Resonator
Pronunciation link: <https://www.merriam-webster.com/dictionary/resonator>
IPA: /ˈrez.əˌnei.t̬ər/
Phonetic Spelling: rezˌuhˌnayˌter
9. Gigahertz
Pronunciation link: <https://www.merriam-webster.com/dictionary/gigahertz>
IPA: /ˈgɪɡ.əˌhɜːts/
Phonetic Spelling: gigˌuhˌhurts
10. Millitesla
Pronunciation link: <https://www.howtopronounce.com/millitesla>
IPA: /ˌmɪl.iˈtes.lə/
Phonetic Spelling: milˌihˌtesˌluh
11. Microtesla
Pronunciation link: <https://www.howtopronounce.com/microtesla>
IPA: /ˌmaɪ.kroʊˈtes.lə/
Phonetic Spelling: myˌkrohˌtesˌluh

12. Multiharmonic
Pronunciation link: <https://www.howtopronounce.com/multiharmonic>
IPA: /ˌmʌl.ti.hɑːrˈmɑː.nɪk/
Phonetic Spelling: mul·tee·har·mon·ik
13. Dispersion
Pronunciation link: <https://www.merriam-webster.com/dictionary/dispersion>
IPA: /dɪˈspɜː.ʒən/
Phonetic Spelling: dih·spur·zhuhn
14. Harmonics
Pronunciation link: <https://www.merriam-webster.com/dictionary/harmonic>
IPA: /hɑːrˈmɑː.nɪks/
Phonetic Spelling: har·mon·iks
15. Absorption
Pronunciation link: <https://www.merriam-webster.com/dictionary/absorption>
IPA: /əbˈzɔːr.pʃən/
Phonetic Spelling: ub·zorp·shuhn
16. Outlier
Pronunciation link: <https://www.merriam-webster.com/dictionary/outlier>
IPA: /ˈaʊt.laɪ.ə/
Phonetic Spelling: out·ly·er
17. Biological replicates
Pronunciation link: <https://www.merriam-webster.com/dictionary/replicate>
IPA: /ˌbaɪ.əˈlɑː.dʒɪ.kəl ˈrep.lə.kəts/
Phonetic Spelling: bye·uh·loj·ih·kuhl rep·luh·kuhts
18. Engraftment
Pronunciation link: <https://www.merriam-webster.com/dictionary/engraftment>
IPA: /ɪnˈgræft.mənt/
Phonetic Spelling: en·graft·muhnt
19. Xenografts
Pronunciation link: <https://www.merriam-webster.com/dictionary/xenograft>
IPA: /ˈziː.nə.græft/
Phonetic Spelling: zee·nuh·graft
20. Albino
Pronunciation link: <https://www.merriam-webster.com/dictionary/albino>
IPA: /ælˈbiː.noʊ/
Phonetic Spelling: al·bee·noh