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Title: Using immunofluorescence to detect PM2.5-induced DNA damage in zebrafish embryo hearts

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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? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   Yes, we can.

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 21
Number of Shots: 39

Introduction

1. Introductory Interview Statements

NOTE to VO talent: Please record the introduction and conclusion statements as well.

REQUIRED:

1.1. This method can be used to accurately and easily detect the expression of related proteins in the zebrafish heart. The changes in the target protein are directly detected by measuring immunofluorescence.

1.1.1. *3.10.1 – 3.10.3.*

Introduction of Demonstrator on Camera

1.2. Demonstrating the procedure will be Yizhou Tao, a master student from the Chen laboratory.

1.2.1. INTERVIEW: Author saying the above.

1.2.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.3. Procedures involving animal subjects have been approved by the Animal Care Institution of The Ethics Committee of Soochow University.

Protocol

2. Morphological observation of zebrafish embryos and cardiac dissection

2.1. Perform zebrafish embryo collection and treatment as described in the text manuscript. At 72 hours post fertilization, transfer the embryos to glass slides **[1]** and observe them under a stereo microscope **[2]**. Record heart malformations, such as pericardial edema, altered looping, and decreased size **[3]**.

2.1.1. Talent transferring an embryo to a slide.

2.1.2. Talent observing embryos under a microscope.

2.1.3. SCOPE: Embryo with heart malformations.

2.2. Calculate malformation rates and analyze differences between groups using one-way ANOVA followed by Turkey's Multiple Comparison Test **[1]**.

2.2.1. Talent at the computer performing statistical analysis.

2.3. After anesthetizing the embryos with 0.6 milligrams per milliliter MS-222, record heart beats for 30 seconds and quantify heart rates using ImageJ software **[1]**.

2.3.1. Talent recording heart beats.

2.4. Carefully dissect hearts from the zebrafish embryos with a disposable syringe needle under a stereo microscope **[1]**. *Videographer: This step is difficult and important!*

2.4.1. SCOPE: Talent dissecting the heart from an embryo.

3. Immunofluorescence Assay

3.1. Use a hydrophobic barrier pen to draw a circle on a clean glass side **[1]**. Add 50 microliters of 4% paraformaldehyde to 1.25 milliliters of PBS to make a fixative solution **[2]**, then place 3 dissected hearts into one hydrophobic barrier circle and incubate for 20 minutes at room temperature **[3]**. *Videographer: This step is important!*

3.1.1. Talent drawing the circle on the slides.

3.1.2. Talent adding the PFA to the circle.

3.1.3. Talent placing the hearts in the PFA.

3.2. Decant the solution under the microscope and dry the samples at room temperature for at least 5 minutes **[1]**. *Videographer: This step is important!*

3.2.1. SCOPE: Talent decanting the solution.

3.3. Wash the slides three times in PBST for 5 minutes per wash **[1]**. Add 50 microliters of BSA to 1000 microliters of PBST to obtain a 5% BSA solution **[2]** and incubate the slides

in a humid chamber for 1 hour to block non-specific antibody binding **[3]**. *Videographer: This step is important!*

3.3.1. Talent washing the slides. **TEXT: PBST: PBS with 0.1% Tween 20**

3.3.2. Talent adding the BSA solution to the slides.

3.3.3. Talent putting the slides in the incubator.

3.4. Decant the solution **[1]** and wash the samples three times with PBS for 5 minutes per wash **[2]**. Dilute 2 microliters of mouse monoclonal antibody against 8-OHdG *(pronounce '8- hydroxy deoxyguanosine')* and 2 microliters of rabbit polyclonal antibody against γH2AX *(pronounce 'gamma-H-2-A-X')* in 296 microliters of PBST to obtain a working primary antibody cocktail solution **[3]**. *Videographer: This step is important!*

3.4.1. Talent decanting the solution.

3.4.2. Talent washing the samples.

3.4.3. Talent diluting the antibodies.

3.5. Incubate the heart samples with 50 microliters of the primary antibody cocktail solution **[1]** in a humidified chamber for at least one hour at room temperature or overnight at 4 degrees Celsius **[2]**.

3.5.1. Talent adding the antibody cocktail to a slide.

3.5.2. Talent putting the slides in the refrigerator.

3.6. Decant the solution **[1]** and wash the samples three times with PBST for 5 minutes per wash **[2]**.

3.6.1. Talent decanting the solution.

3.6.2. Talent washing the samples in PBS.

3.7. Dilute 1 microliter of FITC-labeled goat anti-mouse secondary antibody and 1 microliter of cy3 *(pronounce 'sigh-3')* goat anti-rabbit secondary antibody in 500 microliters of PBST to obtain a working secondary antibody cocktail solution **[1]**.

3.7.1. Talent diluting the antibodies.

3.8. Incubate the samples with the secondary antibodies for 1 hour at room temperature in the dark **[1]**.

3.8.1. Talent adding secondary antibodies to the samples.

3.9. Decant the solution and wash the samples three times with PBS for 5 minutes per wash **[1-TXT]**. Add 20 microliters of DAPI to the samples for nuclear staining and incubate them for 30 minutes at room temperature **[2]**.

3.9.1. Talent washing the sample in PBS. **TEXT: Protect samples from light**

3.9.2. Talent adding DAPI to the samples.

3.10. Apply a coverslip to the slide **[1]** and seal it with nail polish to prevent drying and movement **[2]**. Image the samples under a fluorescence microscope and quantify the fluorescence signal of the heart area using ImageJ software **[3]**.

3.10.1. Talent putting a coverslip on the slide.

3.10.2. Talent sealing the coverslip with nail polish.

3.10.3. Talent at the microscope, imaging the samples.

3.11. Calculate the relative changes using the average of the DMSO control samples and determine the statistical significance of the data **[1]**.

3.11.1. Talent at the computer analyzing the data.

Results

4. Results: Cardiac defects and DNA damage of zebrafish embryos at 72 hpf

4.1. Embryos exposed to $PM_{2.5}$ *(pronounce 'P-M-2.5')* in the absence or presence of the antioxidant N-acetyl-L-cysteine, or NAC, were evaluated for the presence of heart malformations **[1]**.

4.1.1. LAB MEDIA: Figure 1 A.

4.2. EOM from $PM_{2.5}$ caused a significant increase in cardiac teratogenesis such as pericardial edema, altered looping, and decreased size, compared to DMSO control-treated hearts **[1]**.

4.2.1. LAB MEDIA: Figure 1 A. *Video Editor: Emphasize the EOM images.*

4.3. The heartrate was also significantly decreased in embryos exposed to EOM **[1]**. Addition of NAC attenuated EOM-induced heart defects **[2]**.

4.3.1. LAB MEDIA: Figure 1 B. *Video Editor: Emphasize the EOM bar in the heartrate graph.*

4.3.2. LAB MEDIA: Figure 1 B. *Video Editor: Emphasize the EOM + NAC bar both graphs.*

4.4. An immunofluorescence assay was used to evaluate the extent of DNA damage in EOM-treated tissues **[1]**. The levels of 8-OHdG and γH2AX *(pronounce '8- hydroxy deoxyguanosine' and 'gamma-H-2-A-X')* expression were significantly increased in the hearts of zebrafish embryos treated with EOM, indicating an increase in oxidative DNA damage and DNA double strand breaks, respectively **[2]**.

4.4.1. LAB MEDIA: Figure 2 A.

4.4.2. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the EOM column.*

4.5. The EOM-induced DNA damage was partially counteracted by NAC supplementation **[1]**.

4.5.1. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize the EOM + NAC bar in both graphs.*

Conclusion

5. Conclusion Interview Statements

5.1. When attempting this protocol, take care when washing the samples with PBS because the zebrafish heart may peel off the slide.

5.1.1. *3.4.2.*