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Title: Studying Copper Nanoparticle-Induced Programmed Cell Death in Bacteria

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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? **No**

3. Filming location: Will the filming need to take place in multiple locations? **Yes, different floors of the same building.**

Current Protocol Length
Number of Steps: 21
Number of Shots: 39

Introduction

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

1.1. **Betty Revon Liu:** This research investigates copper nanoparticle bactericidal efficacy against three common clinically significant bacteria. It explores mechanisms including ROS generation and potential programmed cell death involvement.

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

What are the current experimental challenges?

1.2. **Holly Liu:** One current challenge is achieving consistent colony counts; I believe this stems from insufficient homogenization during dilution steps, affecting replication compared to more experienced colleagues.

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

What significant findings have you established in your field?

1.3. **Jonathan Wijaya:** Our group was the first to use a cell modulator to study copper nanoparticle bactericidal mechanisms, revealing bacterial death involves cellular processes like ROS generation and autophagy.

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

What research gap are you addressing with your protocol?

1.4. **Meng-Jiun Lai:** The protocol addresses the need for a deeper understanding of the mechanism by which copper nanoparticles kill bacteria, particularly focusing on the under-explored area of bacterial programmed cell death pathways.

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

What advantage does your protocol offer compared to other techniques?

1.5. **Jonathan Wijaya:** Using different cell modulators, we confirmed copper nanoparticle-induced bacterial death involves autophagy and ROS overload, and identified key pathways, supported by reliable, triplicate-based experiments with a simple design.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.3*

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

Protocol

2. Preparation of Copper Nanoparticles and Bacterial Culture Setup

Demonstrator: Jonathan Wijaya

2.1. To begin, obtain commercial copper nanopowders with diameters of 25 nanometers and 60 to 80 nanometers from a commercial supplier **[1]**. Use 1 milliliter of 1 millimolar SDS as a dispersant for each nanoparticle size **[2]**.

2.1.1. WIDE: Talent holding copper nanopowders labeled with 25 nm and 60–80 nm particle sizes.

2.1.2. Talent adding SDS into separate nanoparticle suspension tubes.

2.2. Then disperse the nanoparticles using an ultrasonic bath for at least 30 minutes at room temperature **[1]**.

2.2.1. Talent placing the vials into an ultrasonic bath and the machine operating.

2.3. Next, obtain *Escherichia coli*, *Acinetobacter baumannii*, and St*aphylococcus aureus* strains from respective biological resource centers **[1-TXT]**. Culture each bacteria in 10 milliliters of LB broth under aerobic conditions at 37 degrees Celsius **[2]**.

2.3.1. Shot of labeled culture vials of the three bacterial strains.

AND

TEXT ON PLAIN BACKGROUND:
Escherichia coli (Migula) Castellani and Chalmers strain 25922

Acinetobacter baumannii, Bouvet and Grimont strain: The American Type Culture Collection

Obtain St*aphylococcus aureus* from the Bioresource Collection and Research Center

Video Editor: Please play both shots side by side

2.3.2. Talent transferring cultures into LB broth.

2.4. Dilute bacterial cultures in LB *(L-B)* medium to reach an optical density of approximately 0.5 at 600 nanometers **[1]**. Now, use stock copper nanoparticle solutions to prepare a range of concentrations between 0 to 100 micrograms per milliliter, of both sizes **[2-TXT]**.

2.4.1. Talent diluting bacterial cultures with LB broth.

2.4.2. Talent pipetting of CuNP stock into multiple labeled tubes to create a concentration gradient.

AND

TEXT ON PLAIN BACKGROUND:
Concentration: Reagent Volume

0 µg/mL: Only 500 µL SDS
1 µg/mL: 0.5 µL stock + 499.5 µL SDS
5 µg/mL: 2.5 µL stock + 497.5 µL SDS
10 µg/mL: 5 µL stock + 495 µL SDS
50 µg/mL: 25 µL stock + 475 µL SDS
100 µg/mL: 50 µL stock + 450 µL SDS

Video Editor: Please play both shots side by side

2.5. Pipette 500 microliters of the bacterial cultures into microcentrifuge tubes **[1]** and centrifuge at 3300 *g* for 10 minutes at room temperature **[2]**.
2.5.1. Talent aliquoting bacterial cultures into microcentrifuge tubes.
2.5.2. Shot of tubes being loaded into a centrifuge.

2.6. After pipetting out the supernatant, add different concentrations of both sizes of the nanoparticles to each tube **[1]**. Treat control groups with 500 microliters of PBS as a negative control and 500 microliters of 70% alcohol as a positive control **[2]**. Then incubate all samples with shaking at 200 revolutions per minute at 37 degrees Celsius for 24 hours **[3]**.
2.6.1. Talent pipetting of CuNP suspensions into tubes containing bacterial pellets.
2.6.2. Talent pipetting PBS and 70% alcohol into labelled control sample tubes.
2.6.3. Talent placing the tubes on a shaking incubator set at 200 rpm and 37 °C.

2.7. After incubation, wash the bacteria with PBS **[1]** and spread them onto LB agar plates **[2]**. Incubate the plates at 37 degrees Celsius for 24 hours **[3]**.
2.7.1. Talent pipetting PBS into the tubes.
2.7.2. Shot of bacteria being spread on labeled LB agar plates.
2.7.3. Shot of plates being placed into an incubator set at 37 °C.

2.8. The next day, count colonies on each plate for all treatment groups **[1-TXT]**.
2.8.1. Talent counting colonies. **TXT: Perform statistical analysis on the results**

3. Bactericidal Mechanism Study

3.1. Treat bacteria with 5 micromolar SBI *(S-B-I)* for 2 hours, 0.5 micromolar necrosulfonamide for 1 hour, 100 nanomolar wortmannin for 30 minutes, or 100 nanomolar Z-VAD *(Z-Vad)* for 30 minutes **[1]**.
3.1.1. Talent pipetting of chemical modulators into bacterial tubes labeled with respective concentrations.
AND
TEXT ON PLAIN BACKGROUND:
a. 800µL PBS (for no inhibitor)
b. 700µL PBS + 100µL 5µM SBI (SBI-0206965)

c. 700µL PBS + 100µL 100nM Wort (wortmannin)
d. 700µL PBS + 100µL 0.5µM NSA (necrosulfonamide)
e. 700µLPBS + 100µL 100nM Z-VAD (Z-VAD-FMK)
Video Editor: Please play both shots side by side

3.2. After all pretreatment is complete, centrifuge all tubes at 3300 *g* for 10 minutes **[1].** Then resuspend the pellet in 800 microliters of PBS to wash the pre-treatment modulator **[2].** Distribute the resuspended pellets **[3].**
3.2.1. Talent placing the tubes in a centrifuge.
3.2.2. Shot of the supernatant being pipetted out.
Videographer's Note: Use 3.2.2.1 for talent resuspending pellet in PBS
3.2.3. Shot of the resuspended pellets being distributed.

3.3. Centrifuge each tube then discard supernatant **[1].** Resuspend the pellets in nanoparticle-modulator mixtures before incubating with agitation **[2].**
3.3.1. Shot of supernatant being pipetted out.
Videographer's Note: Use 3.3.1.1for talent placing suspended pellets in centrifuge

3.3.2. Talent resuspending the pellet and placing it on shaker
Videographer's Note: Use 3.3.2.1for talent resuspending pellet

3.4. Now add 70% ethanol and PBS as positive and negative controls **[1]**. Include blank control groups treated with inhibitors but no nanoparticles **[2-TXT]**.
Videographer's Note: 3.4.1-3.4.32 were shot together. Slated as 3.4.1
3.4.1. Talent adding 70% ethanol and PBS to the tubes.
3.4.2. Shot of blank control. **TXT: Incubate for 24 hours more**

3.5. After incubation, pipette the cell viability reagent at a 1 to 10 volume ratio and incubate **[1-TXT].**
3.5.1. Talent pipetting of reagent into the tubes. **TXT: Incubation: 2 h, 37 °C**

3.6. After centrifuging the cultures again, transfer the supernatants to 96-well plates **[1]**. Measure fluorescence at 560 nanometers excitation and 590 nanometers emission using a microplate reader **[2]**.
3.6.1. Talent transferring supernatants into a 96-well plate.
3.6.2. Talent placing the plate in a microplate reader and setting parameters.

3.7. Dilute remaining supernatants to 10^{-5} and 10^{-4} **[1]** and spread on LB agar plates to culture **[2-TXT]**. Count the single colonies the following day **[3]**.
3.7.1. Shot of supernatants being diluted.

3.7.2. Shot of the diluted suspension being spread on LB agar plate. **TXT: Repeat dilution and spreading**
3.7.3. Shot of distinct colony formations on plates.

4. Detection of Reactive Oxygen Species

4.1. Pipette bacterial cultures into microcentrifuge tubes **[1]**. Then expose the cultures to 405 nanometer ultraviolet light for 3 hours **[2]**.
4.1.1. Shot of bacterial cultures being pipetted into microcentrifuge tubes.
4.1.2. Shot of the cultures being placed under UV light.

4.2. Next, incubate the cultures at 45 degrees Celsius for 2 hours **[1]**. Then incubate the bacteria at 4 degrees Celsius for 2 hours **[2]**.
4.2.1. Talent placing the tubes in an incubator.
4.2.2. Talent placing the samples at 4 °C.

4.3. After cold treatment, incubate the bacteria in 3% hydrogen peroxide for 30 minutes **[1]**. Maintain a control group at 37 degrees Celsius in LB broth **[2]**.
4.3.1. Shot of H_2O_2 being added to bacterial suspension.
4.3.2. Shot of labelled tube.

4.4. Now treat bacteria with 20 or 60 nanometer particles at concentrations between 1 to 100 micrograms per milliliter, for 24 hours **[1]**. Then wash the treated bacteria twice with PBS **[2]**.
4.4.1. Shot of bacteria being added into tubes with CuNP.
4.4.2. Talent pipettes PBS into the tubes.

4.5. After centrifugation, resuspend the bacterial pellets in a 5-micromolar solution of 2′,7′-dichlorodihydrofluorescein diacetate dye **[1]**. Analyze the fluorescence intensity at 520 or 530 nanometer emission **[2]**.
4.5.1. Shot of dye being pipetted into the pellets.
4.5.2. Shot of the dyed samples being placed in a flow cytometer and use software to analyze samples.

Results

5. Results

5.1. The colony counts of *Escherichia coli* were significantly reduced by 20 nanometer copper nanoparticles at 1 microgram per milliliter and by 60 nanometer copper nanoparticles at 5 micrograms per milliliter **[1]**. *Staphylococcus aureus* showed significant reductions in colony counts at all concentrations of both nanoparticle sizes **[2]**.

5.1.1. LAB MEDIA: Figure 1A. *Video editor: Please highlight the 1 µg/mL point of black curve and 5µg/mL point of white curve*

5.1.2. LAB MEDIA: Figure 1B.

5.2. In *Acinetobacter baumannii*, reductions in colony numbers required 5 micrograms per milliliter of 20 nanometer copper nanoparticles and 10 micrograms per milliliter of 60 nanometer copper nanoparticles **[1]**.

5.2.1. LAB MEDIA: Figure 1C. *Video editor: Please highlight the 5 µg/mL point of black curve and 10µg/mL point of white curve*

5.3. Copper nanoparticle treatments induced reactive oxygen species production in all bacteria, with 20 nanometer particles showing the highest fractions of positive cells at lower concentrations **[1]**. In contrast, 60 nanometer copper nanoparticle treatments led to consistent reactive oxygen species generation across all concentrations **[2]**.

5.3.1. LAB MEDIA: Figure 2A.

5.3.2. LAB MEDIA: Figure 2B.

5.4. Programmed cell death modulator Z-VAD increased survival of *Escherichia coli* treated with both nanoparticle sizes at low to moderate concentrations **[1]**. NSA treatment improved survival of *Staphylococcus aureus* across all 20 nanometer copper nanoparticle concentrations **[2]**.

5.4.1. LAB MEDIA: Figure 3A and 3B. Video editor: *Highlight Z-VAD bars at 1 to 10 micrograms per milliliter*

5.4.2. LAB MEDIA: Figure 3C. *Video editor: Please highlight the NSA-patterned bars*

5.5. *Acinetobacter baumannii* exhibited improved viability with Z-VAD and NSA treatments under 20 and 60 nanometer copper nanoparticle exposure **[1]**.

5.5.1. LAB MEDIA: Figure 3E and 3F. *Video editor: Please emphasize taller Z-VAD and NSA bars in both figures*

Pronunciation Guide:

1. Programmed Cell Death
Pronunciation link: No confirmed link found
IPA: /ˈproʊˌgræmd sɛl dɛθ/
Phonetic Spelling: proh-gramd sel deth

2. Autophagy
https://www.merriam-webster.com/dictionary/autophagy
IPA: /ˈɔː.təˌfeɪ.dʒi/
Phonetic Spelling: aw-tuh-fay-jee

3. *Acinetobacter baumannii*
https://www.howtopronounce.com/acinetobacter-baumannii
IPA: /ˌæs.ɪˈniː.təˌbæk.tər ˈbɔː.mɑː.ni.aɪ/
Phonetic Spelling: ass-ih-nee-tuh-bak-ter baw-mah-nee-eye

4. Necrosulfonamide
Pronunciation link: No confirmed link found
IPA: /ˌnɛk.roʊˈsʌl.foʊ.nəˌmaɪd/
Phonetic Spelling: nek-roh-suhl-foh-nuh-myd

5. Wortmannin
Pronunciation link: No confirmed link found
IPA: /ˈwɝː.mæn.ɪn/
Phonetic Spelling: wert-man-in

6. Z-VAD
Pronunciation link: No confirmed link found
IPA: /zi væd/
Phonetic Spelling: zee-vad

7. 2’,7’-Dichlorodihydrofluorescein Diacetate
Pronunciation link: No confirmed link found
IPA: /daɪˌklɔː.roʊ.daɪˌhaɪ.drəˈflɔː.rəˌsin ˌdaɪ.əˈsɛ.teɪt/
Phonetic Spelling: dye-klor-oh-dye-hy-dro-flor-uh-seen dye-uh-seh-tayt

8. Reactive Oxygen Species
No confirmed link for full phrase
IPA: /riˈæk.tɪv ˈɑːk.sɪ.dʒən ˈspiː.ʃiz/
Phonetic Spelling: ree-ak-tiv aak-sih-jin spee-sheez

9. Microplate Reader
Pronunciation link: No confirmed link found
IPA: /ˈmaɪ.kroʊ.pleɪt ˈriː.dɚ/
Phonetic Spelling: my-kroh-playt ree-dur

10. Colony Forming Unit (CFU)
Pronunciation link: No confirmed link found
IPA: /ˈkɑː.lə.ni ˈfɔːr.mɪŋ ˈjuː.nɪt/
Phonetic Spelling: kah-luh-nee for-ming yoo-nit

11. SDS (Sodium Dodecyl Sulfate)
Pronunciation link: No confirmed link found
IPA: /ˌɛs.diːˈɛs/
Phonetic Spelling: ess-dee-ess

12. Hydrogen Peroxide
https://www.merriam-webster.com/dictionary/hydrogen%20peroxide
IPA: /ˈhaɪ.drə.dʒən pəˈrɑːk.saɪd/
Phonetic Spelling: hy-druh-jen puh-rok-syd

13. *Staphylococcus aureus*
https://www.merriam-webster.com/medical/Staphylococcus%20aureus
IPA: /ˌstæf.ɪ.ləˈkɑː.kəs ˈɔːr.i.əs/
Phonetic Spelling: staf-ih-luh-kaw-kus awr-ee-us

14. *Escherichia coli*
https://www.merriam-webster.com/dictionary/Escherichia%20coli
IPA: /ˌɛʃ.əˈrɪk.i.ə ˈkoʊ.laɪ/
Phonetic Spelling: esh-uh-rik-ee-uh koh-ly