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Title: Estimation of urinary nanocrystals in humans using calcium fluorophore labeling and Nanoparticle Tracking Analysis

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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?

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

   NO

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES**

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**.

   X

   Dr. Mitchell stated the introductory and conclusion interview statements on camera.

4. **Filming location:** Will the filming need to take place in multiple locations? **YES**

   If **Yes**, how far apart are the locations? **0.1 mile**

Current Protocol Length

Number of Steps: 17
Number of Shots: 41

Introduction

1. Introductory Interview Statements

REQUIRED:

NOTE: All statements were delivered by Tanecia Mitchell on camera. Suggested shots can be used as B-roll

1.1. Tanecia Mitchell: This protocol can specifically detect calcium containing nanocrystals in human urine.
1.1.1. *2.3.1.*

1.2. Tanecia Mitchell: This technique could be helpful to predict early stages of kidney stone formation or other diseases associated with crystalluria.

1.2.1. *3.10.1.*

Introduction of Demonstrator on Camera NOTE: Dr. Mitchell introduced Dr. Kumar on camera

1.3. Tanecia Mitchell: Demonstrating the procedure will be Dr. Parveen Kumar, a Researcher IV from my laboratory.

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

Ethics Title Card

1.4. All experiments outlined in this work were approved by the University of Alabama at Birmingham (UAB) Institutional Review Board.

Protocol

2. Urine collection and processing

2.1. Have participants consume a low oxalate diet for 3 days and fast overnight **[1]**. Then, collect a 24-hour urine sample from the participants before having them consume an oxalate load **[2-TXT].**

2.1.1. Participants fed low oxalate diet.

2.1.2. Urine sample. **TEXT: Wear PPE and handle human samples with care**

2.2. Feed the participants with a smoothie containing fruits and vegetables having an oxalate load of approximately 8 millimolar **[1].** Have participants collect their urine for 24 hours post-oxalate consumption and return it on the following day **[2].** Maintain all urine samples at room temperature prior to processing **[3].**

Added shot: CU of smoothie

2.2.1. Participants drinking an oxalate loaded smoothie. NOTE: 2.2.1 and 2.2.2 were shot out of sequence

2.2.2. Talent collecting urine sample.

2.2.3. Talent keeping the urine samples in room temperature. NOTE: 2.2.3 – 2.3.2 shot together

2.3. Measure and record urine pH and volume of the urine samples **[1]**. Mix thoroughly **[2]** and add 50 milliliters of urine into a labeled sterile 50-milliliter conical tube **[3].** Centrifuge the sample at 1200 times *g* for 10 minutes at room temperature using a benchtop centrifuge **[4].** *Videographer: This step is important!*

2.3.1. Talent measuring the pH and volume of the urine.

2.3.2. Talent mixing the urine.

2.3.3. Talent transferring the urine into conical tubes. NOTE: CU also available

2.3.4. Talent centrifuging the sample. NOTE: Reuse this shot for Step 2.4.3.

2.4. Discard the supernatant **[1]**, then wash and resuspend the pellet again with 5 milliliters of 100% ethanol **[2]**. Centrifuge the sample at 1200 times *g* for 10 minutes at room temperature using a benchtop centrifuge **[3].** *Videographer: This step is important!*

2.4.1. Talent discarding the supernatant. NOTE: Reuse this shot for Step 2.5.1.

2.4.2. Talent adding 100% ethanol to the tube.

2.4.3. Talent centrifuging the sample. NOTE: Use 2.3.4.

2.5. Discard the supernatant **[1]** and resuspend the pellet in 1 milliliter of 100% ethanol **[2]**. Store the sample at minus 20 degrees Celsius for later processing or immediately proceed with staining the samples **[3].** *Videographer: This step is important!*

2.5.1. Talent discarding the supernatant. NOTE: Use 2.4.1

2.5.2. Talent adding 100% ethanol to the pellet.

2.5.3. Talent storing the sample in the freezer.

3. Nanoparticle Tracking Analysis (NTA)

3.1. Dilute 100-nanometer sized gold nanoparticles in ultrapure water at a ratio of 1 to 1000 and use them for optimizing the settings on the instrument **[1].** Dilute the urine samples 20 times in water prior to staining with 5 millimolar Fluo-4 AM *(Flr-Oh-4-A-M)* for 30 minutes in the dark and analyze the samples using NTA **[2].** *Videographer: This step is important!*

3.1.1. Talent diluting the gold nanoparticles for instrument optimization. NOTE: This and next shot combined and out of sequence

3.1.2. Talent diluting urine sample.

3.2. Prepare calcium oxalate and calcium phosphate crystals as described in the text manuscript prior to NTA analysis **[1]**. NOTE: Please add this VO to the following shot 3.3.1 because 3.2.1. was not recorded

~~3.2.1. This step was not recorded. It was not necessary to show crystals being weighed on a scale.~~

3.3. Turn on the computer and the instrument **[1]**, then open the software and turn on the camera **[2].**

3.3.1. Talent turning on the computer and the instrument.

3.3.2. LAB MEDIA: Jove62192_3.3.2.mp4. 00:01-00:12.

3.4. Click the capture icon in the top left corner of the window to start the capture mode **[1]**.

3.4.1. LAB MEDIA: Jove62192_3.4.1.mp4. 00:01-00:17.

3.5. Clean the platform by first pumping air into it using a 1-milliliter syringe until the platform appears clean **[1]**. Gently add water to the apparatus 2 to 3 times **[2]** and use another 1-milliliter syringe to remove any air bubbles **[3]**. *Videographer: This step is difficult and important!*

3.5.1. Talent pumping air into the platform using syringe. NOTE: 3.5.1 – 3.5.3 shot together

3.5.2. Talent adding water to the apparatus.

3.5.3. Talent removing air bubbles.

3.6. Once the platform is clean, add water and check for any contamination on the surface by viewing the camera **[1]**, then add gold nanoparticles as a control to the sample loading pump injector to set up the instrument **[2].**

3.6.1. Talent adding water to check contamination on the surface of the instrument.

3.6.2. Talent adding gold nanoparticles to the sample loading pump injector. NOTE: CU also available

3.7. Adjust the camera level on the screen or on the knob to the right side of the instrument until the image starts to display colored pixels, then reduce the camera level **[1]**.

3.7.1. LAB MEDIA: Jove62192_3.7.1.mp4. 00:10-00:35.

3.8. Next, adjust the screen to optimize the image. **Left-click** the mouse button on the video image. **Hold** the **left mouse button** and **drag** the image up and down to get the entire view **[1].**

3.8.1. LAB MEDIA: Jove62192_3.8.1.mp4. 00:06-00:20.

3.9. Set up the infusion speed and focus the camera so that the gold nanoparticles are visible on the camera screen. Set the infusion speed to high for initial set up to ensure the gold nanoparticles are detected **[1]**. Once detected, reduce the speed to 50 microliters per minute to visualize gold nanoparticles **[2].**

3.9.1. LAB MEDIA: Jove62192_3.9.1.mp4. 00:11-00:18.

3.9.2. LAB MEDIA: Jove62192_3.9.2.mp4. 00:01-00:10.

3.10. Adjust the camera level to visualize the particles **[1]**. For unstained samples, adjust the screen gain at level 5 to achieve the camera focus, and set the camera level at 8. Once the focus is set, record the sample **[2].**

3.10.1. LAB MEDIA: Jove62192_3.10.2_t2.mp4. 00:01-00:20.

3.10.2. LAB MEDIA: Jove62192_3.10.1.mp4. 00:01-00:24.

3.11. After optimization, clean the apparatus again with water before assessing other samples to ensure that the tubing is clean and particles are not present **[1].**

3.11.1. Talent cleaning the apparatus with water.

3.12. To analyze stained samples, adjust the camera to the filter position containing the suitable fluorescent filter **[1]** and load diluted and stained samples onto the sample loading pump injector **[2]**, reducing the speed to 20 microliters per minute for analysis **[3].** Adjust the screen gain to 5 and camera level to 13 **[4-TXT].**

3.12.1. LAB MEDIA: Jove62192_3.13.1_t1.mp4. 00:01-00:14.

3.12.2. Talent loading the diluted stained sample onto the sample loading pump injector.

3.12.3. LAB MEDIA: Jove62192_3.12.4.mp4. 00:01-00:10.

3.12.4. LAB MEDIA: Jove62192_3.12.3.mp4. 00:01-00:30. **TEXT: Take 5 captures/sample of 60 s duration**

3.13. Save the data after each measurement **[1]**. Calculate the average number of nanoparticles for all 5 readings for each individual sample and analyze the data using standard deviation or standard error of the mean. Use t-tests for paired analysis **[2].**

3.13.1. LAB MEDIA: Jove62192_3.13.1_t2.mp4. 00:01-1:55. *Video editor please speedup the video and play.*

3.13.2. LAB MEDIA: Jove62192_3.13.2.mp4. 00:01-1:13. *Video editor please speedup the video and play.*

Results

4. Analysis of NTA detecting Fluo-4 AM labelled CaOx and CaP in 24-hour human urinary nanocrystals

4.1. Fluo-4 AM was able to bind to both calcium oxalate crystals, which were between 50 and 270 nanometers in size and had a mean concentration of 1,260 million particles per milliliter **[1]** and calcium phosphate crystals, which were between 30 and 225 nanometers and had a mean concentration of 2,220 million particles per milliliter **[2]**.

4.1.1. LAB MEDIA: Figure 3A.

4.1.2. LAB MEDIA: Figure 3B.

4.2. Twenty-four-hour urine samples collected before the oxalate load contained some urinary nanocrystals between 110 to 300 nanometers in size **[1].** In contrast, there was a significant increase in urinary nanocrystals present in post-oxalate samples **[2].**

4.2.1. LAB MEDIA: Figure 4. *Video editor focus on the small blue line graph.*

4.2.2. LAB MEDIA: Figure 4. *Video editor focus on the big red line graph.*

4.3. To confirm the reproducibility of the method, samples were measured three times and there was no significant variation in technical replicates **[1].**

4.3.1. LAB MEDIA: Figure 5.

Conclusion

5. Conclusion Interview Statements

NOTE: All statements were delivered by Tanecia Mitchell on camera. Suggested shots can be used as B-roll

5.1. Tanecia Mitchell: When attempting this protocol, there may be some difficulty adding samples without bubbles to the machine. To prevent this, add air and water before adding samples slowly to the apparatus. Make sure to continually check for bubbles while measuring samples.

5.1.1. *3.5.*

5.2. Tanecia Mitchell: This new method will be further tested to monitor disease progression and/or predict kidney stone risk in individuals diagnosed with calcium oxalate kidney stones.

5.2.1. *2.1.2.*