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Scriptwriter Name: Bridget Colvin
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Title: Whole Animal Imaging of *Drosophila melanogaster* Using Microcomputed Tomography

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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 similar? **Y, Leica MZ10F**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y. Done**

Videographer: Film all SCREEN shots as a backup

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**.

☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **39**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Todd Schoborg**: This protocol allows the nondestructive imaging of intact *Drosophila* at a micron-resolution and can be used to understand the mechanisms of animal physiology, anatomy, and development **[1]**.

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

1.2. **Todd Schoborg**: Because the flies are imaged in an intact state without the need for tissue dissection, the spatial architectures of the tissues and organs are preserved in their native state and easily observed **[1]**.

1.2.1. INTERVIEW*:* Named talent says the statement above in an interview-style shot, looking slightly off-camera *B-roll: 3.3.1*

OPTIONAL:

1.3. **Todd Schoborg**: Micro-CT is an ideal tool for studies aimed at modeling human diseases in flies or for understanding the developmental defects that affect quality of life. It has also proven to be useful for studies in engineering, materials science, physics, geology, and anthropology **[1]**.

1.3.1. INTERVIEW*:* Named talent says the statement above in an interview-style shot, looking slightly off-camera *B-roll: 5.1.1*

Protocol

2. Sample Selection, Cuticle Preparation, Fixation, and Staining

2.1. After selecting the developmental timepoint of interest for imaging, transfer 5-50 animals into 1 milliliter of PBST (P-B-S-T) in a 1.5-milliliter microcentrifuge tube **[1-TXT]** and incubate the tube for 5 minutes at room temperature with periodic tapping to assist in the removal of the hydrophobic coating and to allow the animals to become fully submerged **[2]**.

2.1.1. WIDE: Talent adding flies to tube, with PBST container visible in frame **TEXT: PBST: 0.5% Triton X-100 in 1x PBS**

2.1.2. Talent picking up and tapping tube

2.2. At the end of the incubation, replace the PBST with 1 milliliter of Bouin's solution **[1]** and tap the tube to fully submerge the flies **[2]**.

2.2.1. Talent adding Bouin's solution to tube, with Bouin's solution container visible in frame *Videographer: Important step*

2.2.2. Tube being tapped *Videographer: Important step*

2.3. For larval and pupal samples, after a 2-hour incubation at room temperature, wash the animals three times for 5 minutes and 1 milliliter of fresh PBS per wash **[1]**.

2.3.1. Talent adding PBS to tube(s), with PBS container visible in frame

2.4. After the last wash, transfer the samples to a multi-well dissecting dish containing fresh PBS per well under a dissecting microscope **[1]** and use a small minutien pin attached to a holder to poke a hole in the anterior and posterior cuticle of each sample, taking care not to disrupt the underlying soft tissue **[2]**.

2.4.1. Talent adding samples to dish *Videographer: Important step*

2.4.2. SCOPE: Cuticle(s) being poked *Videographer: Important step*

2.5. After penetrating the cuticles, incubate the samples in 1 milliliter of fresh Bouin's solution in a new 1.5-milliliter tube for 24 hours at room temperature **[1]** before washing the samples three times for 30 minutes and 1 milliliter of micro-CT (C-T) wash buffer per wash **[2-TXT]**.

2.5.1. Talent adding Bouin's solution to tube, with Bouin's solution container visible in frame

2.5.2. Micro-CT wash buffer being added to tube, with micro-CT wash buffer container visible in frame **TEXT: Alternative: wash x3 in PBS**

2.6. After the last wash, add 1 milliliter of the appropriate staining solution to the tube **[1]** and incubate the samples at room temperature for 2-7 days **[2]**.

2.6.1. Talent adding solution to tube, with solution container visible in frame *Videographer: Important step*
2.6.2. Talent placing sample onto benchtop *Videographer: Important step*

2.7. At the end of the incubation, wash the samples two times for 30 minutes with 1 milliliter of ultrapure water per wash **[1]** before transferring the samples to a new aliquot of ultrapure water for their storage until scanning **[2-TXT]**.

2.7.1. Talent adding water to tube
2.7.2. Talent transferring sample to tube **TEXT: Alternative: Wash and store in PBS** Authors NOTE: This shot was not filmed

2.8. If longer image preservation is necessary, dehydrate the samples with an ascending ethanol series in 1 milliliter of ethanol for 1 hour per concentration as indicated to prepare the samples for critical point drying **[1-TXT]**.

2.8.1. Talent placing samples into 10% ethanol, with other concentrations visible in frame **TEXT: 10% -> 25% -> 50% -> 75% -> 100% x2**

3. Sample Mounting

3.1. To mount critical point dried samples, place the samples in plastic or glass capillary tubes **[1]** before mounting the tubes onto the rotating stage holder **[2]**.

3.1.1. WIDE: Talent adding samples to capillary tube
3.1.2. Talent mounting tube onto holder

3.2. For hydrated sample mounting, fill a P10 pipette tip with water **[1]** and secure the narrow end with paraffin film **[2-TXT]**.

3.2.1. Talent filling pipette *Videographer: Important/difficult step*
3.2.2. Tip being sealed with paraffin film *Videographer: Important/difficult step* **TEXT: Alternative: Seal with paraffin film**

3.3. Use forceps to transfer a single specimen to the pipette tip **[1]** and use a long, slender object to carefully push the specimen down into tip until it just contacts the wall of the tip to hold the sample in place **[2]**.

3.3.1. Talent placing sample into tip
3.3.2. Sample being pushed into tip

3.4. After covering the open end of the pipette, mount the P10 pipette onto a holder designed to fit within the chuck of the rotating stage **[1]**.

3.4.1. Talent mounting pipette onto holder *Videographer: Important/difficult step*

4. Scanning

4.1. For scanning, click the **Open Door** icon the scanner software to gain access to the rotating stage chuck **[1]** and tighten the collar around the base of the sample holder to attach the sample **[2]**.

4.1.1. WIDE: Talent clicking icon, with monitor visible in frame
4.1.2. Talent tightening collar

4.2. Set the scanning parameters in the software for optimal resolution and contrast **[1]** and click **Options** and **X-ray source** to open the X-ray source power control **[2]**.

4.2.1. Talent setting parameters
4.2.2. SCREEN: 4.2.2.mkv. 0:01-0:04. Options and x-ray source being clicked *Videographer: Film the screen*

4.3. Use the slider bars to set the X-ray voltage to 30-40 kilovolts and the current to 100-110 microamps to produce an X-ray beam with 3-4 Watts of power and a small spot size **[1]**.

4.3.1. SCREEN: 4.3.1.mkv. 0:01-0:17. X-ray voltage being set, then current being set *Video Editor: Feel free to speed up.*

4.4. To set the number of projection images to be acquired during the scans, select **Options** and **Acquisition Modes** to set the camera exposure time to 500-800 milliseconds and use the slider bar to set the desired image pixel size according to the camera settings and position **[1-TXT]**.

4.4.1. SCREEN: : 4.4.1.mkv. 0:01-0:22. Options and Acquisitions mode being selected, camera exposure time being set, pixel size being set **TEXT: More projections = enhanced resolution but longer scan time** *Video Editor: Emphasize the 'Acquisition Modes' tab being pressed at 0:04. Speed up the footage till 0:16. Emphasize 13.11 µm tab being pressed at 0:16.*

4.5. Click and drag the slider bar to move the sample along its 360-degree rotation path, keeping the sample within the field of view **[1]**.

4.5.1. SCREEN: 4.5.1.mkv. 0:01-0:17. Sample being moved along rotation path *Video Editor: Emphasize the slider bar being clicked between 0:04-0:05. Speed up the footage after.*

4.6. Click the **Begin Acquisition** icon. A dialog box will appear allowing additional scanning parameters to be set. Name the file and the output folder to which the scan will be saved **[1]**.

4.6.1. SCREEN: 4.6.1.mkv. 0:01-0:13. Icon being clicked, box appearing, file being named, folder being selected*. Video Editor: Emphasize arrow icon being clicked at 0:01. Videographer: Film the screen*

4.7. Set the random movement to 10 and the average to 4-6 frames. The rotation step will be automatically calculated depending on the camera settings used **[1]**.

4.7.1. SCREEN: 4.7.1.mkv. 0:00-0:05. Random movement. *Videographer: Film the screen*

4.8. Then click **OK** to begin the acquisition. A progress bar dialog box showing the scan time will appear and the scanner will begin acquiring a series of projection images of the specimen along the rotation path **[1]**.

4.8.1. SCREEN: 4.8.1.mkv. 0:02-0:20. Ok being clicked, progress bar dialog box appearing, images being acquired *Video Editor: Emphasize 'OK' tab being clicked at 0:03. Speed the footage after the dialogue box appears. Videographer: Film the screen*

5. Reconstruction and Image Analysis

5.1. To generate the tomograms, open the projection images in the Reconstruction software **[1]** and select the shift-correction algorithm to perform an initial image alignment **[2]**.

5.1.1. WIDE: Talent opening images, with monitor visible in frame

5.1.2. SCREEN: 5.1.2.mkv. 0:00-0:26. *Video Editor: Speed up the footage till the dialogue box appears at 0:20.*

5.2. Select the best value that properly aligns the projection images and click **Fine Tuning** to fine tune each reconstruction parameter. A series of previews will be generated that can be used to select the correct values **[1]**.

5.2.1. SCREEN: 5.2.1.mkv. 0:01-0:40 *Video Editor: Emphasize 'fine tuning' tab being clicked at 0:02. Speed up the footage between 0:07-0:35. Emphasize arrow tab being clicked at 0:38.*

5.3. Confirm that the optimal parameter values are displayed in the **Settings** tab and, in the **Output** tab, adjust the file parameters, such as the bit-depth, file name, and folder where in which the data will be saved **[2]**.

5.3.1. SCREEN: 5.3.1.mkv. 0:00-0:09. Clicking on Settings tab. *Video Editor: Emphasize 'Settings' tab being clicked at 0:02. Videographer: Film the screen*

5.4. A region of interest can be used to reconstruct only the structures of interest **[1]**.

5.4.1. Screen: 5.4.1.mkv. 0:02-0:35. clicking on the output tab and adjusting file parameters *Video Editor: Emphasize 'output' tab being clicked at 0:04. Speed up the footage till 0:09. Emphasize 'use ROI' tab being clicked at 0:17. Videographer: Film the screen*

5.5. If multiple reconstructions are required, click **Start** and **Add to batch** to add the current image to the batch manager **[1]** and repeat the reconstruction for the remaining images **[2]**.

5.5.1. SCREEN: 5.5.1.mkv. 0:01-0:08. Start and add to batch being clicked *Video Editor: Emphasize 'Start' tab being clicked at 0:02 and 'Add to batch' being clicked at 0:06. Videographer: Film the screen*

5.5.2. SCREEN: 5.5.2.mkv. 0:02-0:09. Image being opened and/or Start and Start being clicked *Video Editor: Emphasize 'start batch' icon being clicked at 0:03 and speed up the footage after. Videographer: Film the screen*

5.6. For morphometric analysis of the reconstructed images, under the **Segment** tab in an appropriate morphometric analysis software program, click **Basic** and **New**, give the image a new name, and select an appropriate color **[1]**.

5.6.1. SCREEN: 5.6.1.mkv. 0:01- 0:16. Basic and New being clicked, image being named, then color being selected *Video Editor: Emphasize 'Segment' icon being clicked at 0:03 and 'New' tab being at 0:05. clicked Videographer: Film the screen*

5.7. Check the **Define Range** box and adjust the slider to adjust the threshold range that encompasses the structure of interest **[1]**.

5.7.1. SCREEN: 5.7.1.mkv. 0:01-0:10. Clicking Define Range box and adjusting slider. *Videographer: Film the screen*

5.8. To paint an area defined by the threshold, press and hold the **Ctrl** key while holding the left mouse key. To remove an area, press and hold the **Shift** key while holding the left mouse key **[1]**.

5.8.1. SCREEN: 5.8.1.mkv. 0:01-0:33. Area being painted, then area being removed *Video Editor: Feel free to speed up the footage. Videographer: Film the screen*

5.9. Ensure that the measurements of the mesh region of interest are displayed in the **Information** panel for basic morphometric analysis **[1-TXT]**.

5.9.1. SCREEN: 5.9.1.mkv. 0:00-0:09. Shot of measurements in Information panel **TEXT: Use Object Analysis Module for additional analyses** *Video Editor: Emphasize 'surface' and volume' boxes being checked and 'Refresh' tab pressed between 0:02-0:05. Videographer: Film the screen*

5.10. Render the mesh object and the entire tomogram image and visualize the resulting image in 3D **[1]**.

5.10.1. SCREEN: 5.10.1.mkv. 0:01-0:26. Mesh object and entire tomogram being rendered, then visualized in 3D *Video Editor: Emphasize 'Edge contrast' box being checked at 0:09, '3D' icon clicked at 0:12 and mesh icon clicked at 0:14. Speed up the footage after 0:19. Videographer: Film the screen*

5.11. Then right click to select **Show Movie Maker** to generate a video of the object using individual frames from the viewer **[1]**.

5.11.1. SCREEN: 5.11.1.mkv. 0:01-0:55. Show Movie Maker being selected/video being generated *Video Editor: Speed up the footage after 'Save' icon is clicked at 0:38. Videographer: Film the screen*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.2., 2.4., 2.6., 3.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.2., 3.4. A custom-built holder that fits into the rotating stage chuck and has a small indention to fit the end of the pipet tip and hold it straight is extremely helpful.

Results

6. Results: Representative *Drosophila melanogaster* Imaging

6.1. In these images **[1]**, an embryo **[2]**, 3rd instar larva **[3]**, pupa at the pharate adult stage **[4]**, and adult female fly stained with iodine and imaged in water using a commercial benchtop scanner can be observed **[5]**.

6.1.1. LAB MEDIA: Figure 2
6.1.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2A*
6.1.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2B*
6.1.4. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2C*
6.1.5. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2D*

6.2. Excellent preservation and even staining of the delicate tissue are apparent, allowing all of the major organs to be readily identified **[1]**.

6.2.1. LAB MEDIA: Figure 2 *Video Editor: please some organ texts and lines or no animation*

6.3. Here a comparison of the same adult fly headcase **[1]** acquired in both slow **[2]** and fast projection scan mode is shown **[3]**.

6.3.1. LAB MEDIA: Figures 3
6.3.2. LAB MEDIA: Figures 3 *Video Editor: please emphasize right column of images*
6.3.3. LAB MEDIA: Figures 3 *Video Editor: please emphasize left column of images*

6.4. Importantly, although the resolution in the slow images is higher **[1]**, the morphometric analysis does not differ between the 'slow' and 'fast' scans **[2]**.

6.4.1. LAB MEDIA: Figures 3
6.4.2. LAB MEDIA: Figure 3 *Video Editor: please add/emphasize ns text and bracket*

6.5. Using the rendering software, any tissue or organ system of interest can be segmented and used for morphometric analysis and 3D visualization **[1]**.

6.5.1. LAB MEDIA: Movie 1: 00:15-00:42 *Video Editor: please speed up*

6.6. In these images **[1]**, a fly abdomen stained with phosphotungstic acid and imaged while water hydrated **[2]** or following critical point drying on an X-ray microscope can be observed **[3]**.

6.6.1. LAB MEDIA: Figure 4
6.6.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize Water column*
6.6.3. LAB MEDIA: Figure 4 *Video Editor: please emphasize CPD column*

6.7. Using phosphotungstic acid, individual epithelial cells of the midgut **[1]** and sperm bundles within the testes are easily resolved **[2]**.

6.7.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize Mg text and lines in Water images*
6.7.2. LAB MEDIA: Figure *Video Editor: please emphasize Te text and lines in Water images*

6.8. While the critical point drying images show marginally increased resolution compared to the hydrated sample **[1]**, better preservation of the ultrastructure of delicate tissues is achieved with hydrated samples such as the fat cells near the cuticle **[2]**.

6.8.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize CPD images*
6.8.2. LAB MEDIA: Figures 4A' and 4B' *Video Editor: please provide side by side close up of FC labelled regions from both images*

Conclusion

7. Conclusion Interview Statements

7.1. **Todd Schoborg**: For the best t resolution, limit sample movement and excess vibrations. Securely mounting the sample to the stage is critical, although creativity and resourcefulness may be required to achieve this **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *B-roll: 3.4*

7.2. **Todd Schoborg**: Additional imaging methods, such as light or electron microscopy, can be incorporated as an 'imaging workflow' that allows the investigation of a given mutation from the nanoscale to the whole organism **[1]**.

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