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Title: Transmission Electron Microscopy as the Visualization Technique for Analysis of Circadian Synaptic Plasticity in the Mouse Barrel Cortex

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

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

Authors: Please create screen capture videos of the shots labeled as SCREEN, create a screenshot summary, and upload the files to your project page as soon as possible: **https://review.jove.com/account/file-uploader?src=20857473**

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Current Protocol Length

Number of Steps: 29
Number of Shots: 54

Introduction

1.1. **Elżbieta Pyza**: We are investigating the molecular mechanisms underlying neuroplasticity in both vertebrates and fruit flies, using wild-type organisms as well as models of neurodegenerative diseases.

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

What are the most recent developments in your field of research?

1.2. **Elżbieta Pyza**: The number of animal models for neurodegenerative diseases is steadily growing, along with the development of methods to investigate the role of neuroplasticity under both normal and pathological conditions.

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. **Elżbieta Pyza**: We have found that the number of synapses and structures of neuron and glia cells change during the day and night, and in neurodegenerative diseases compared to wild-type animals.

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

Ethics Title Card

This research has been approved by the appropriate institutional ethics committee and conducted in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, as well as with national regulations.

Protocol

2. Preparation of Brain Tissues for Sectioning

Demonstrator: Małgorzata Jasińska

2.1. To begin, take the mouse's cerebellum and divide the brain into two hemispheres **[1]**. Choose one hemisphere and orient it so that sections can be cut tangentially to the barrel cortex **[2-TXT]**.

2.1.1. WIDE: Talent splitting the brain into two hemispheres.

2.1.2. Talent holding one hemisphere and orienting it in the correct position. **TXT: Mount and glue the hemisphere onto the vibratome chuck**

2.2. Then, cut the sections at 60-micrometer thickness **[1]** and transfer them into a solution containing 0.1 molar phosphate buffer and fixative in a 1 to 5 ratio **[2]**.

2.2.1. Talent cutting the sections.

2.2.2. Talent transferring brain sections into the fixative and buffer mixture.

2.3. Examine the sections under a light microscope and collect only those sections that clearly show the barrel field cortex **[1-TXT]**.

2.3.1. LAB MEDIA: figure 2E **TXT: Use a concave slide to keep the section submerged during observation**

3. Brain Fixation and Sectioning

Demonstrator: Anbarieh Saadat

3.1. Using a paintbrush, gently transfer the sections into a small glass Petri dish **[1]**. Rinse the brain sections in 0.1 molar cacodylate **[2-TXT]**.

3.1.1. Talent using a paintbrush to move brain sections into a glass Petri dish.

3.1.2. Talent rinsing the sections in buffer through three 5-minute cycles. **TXT: Repeat the cycle 3x for 5 min each**

3.2. Then, fix the sections in 1 percent osmium tetroxide in 0.1 molar cacodylate buffer with 1.5 percent potassium ferricyanide **[1-TXT].**

3.2.1. Talent placing the sections in Petri dish with fixative. **TXT: Incubations: +4 °C, 1 h; RT, 1h**

3.3. Once the sections are rinsed in distilled water, use a syringe with a 25-millimeter filter to slowly dispense 70 percent ethanol containing 1 percent uranyl acetate into each Petri dish with tissues **[1-TXT]**. NOTE: The VO is edited for the deleted shot

~~3.3.1. Talent placing Petri dish in ethanol and uranyl acetate solution.~~ NOTE: Not filmed

3.3.2. Talent using a syringe with a filter to pour solution into Petri dishes. **TXT: Dehydrate the tissue in an increasing EtOH concentration**

3.4. After overnight incubation in 70% ethanol at 4 degrees Celsius, wash tissues twice in propylene oxide for 10 minutes each **[1]**.

3.4.1. Talent performing two propylene oxide washes by gently swirling the Petri dish.

3.5. Next, replace propylene oxide with a mixture of 2 milliliters of Polybed resin and propylene oxide **[1].** Cover with a lid and incubate for 40 minutes **[2]**.

3.5.1. Talent pouring resin mixture into Petri dishes.

3.5.2. Talent placing lids on the petri dish and keeping it aside.

3.6. Embed the brain sections in a mixture of 2 milliliters of Polybed resin and propylene oxide, cover, and leave for 1.5 hours **[1]**.

3.6.1. Talent pouring resin mixture and covering the Petri dishes.

3.7. Then, cut the Aclar film into pieces matching the size of a standard glass slide **[1]**. Using a plastic pipette, apply a small volume of resin onto each Aclar film piece **[2]**. With a paintbrush, transfer the brain sections from the Petri dish to the resin on the Aclar film **[3]**. Embed each section between two Aclar films and incubate to polymerize **[4-TXT]**.

3.7.1. Talent cutting Aclar film into slide-sized pieces. NOTE: 3.7.1, 3.7.2, 3.7.3, and 3.7.4 are filmed in one take.

3.7.2. Talent pipetting resin onto the surface of the Aclar film.

3.7.3. Talent placing brain sections on the resin using a paintbrush.

3.7.4. Talent sandwiching sections between two Aclar films. **TXT: Incubation: 65 °C**; **48 h**

4. Barrel Cortex Imaging and Sectioning

4.1. Using a light microscope with a 2x objective, photograph the embedded brain sections

[1].

4.1.1. LAB MEDIA: Figure 2F.

4.2. To open the images, click **File** and **Open**, select the images while holding **Shift**, and click **Open [1]**. Begin stacking images from the cortex. Right-click on **Layer 1** in each image, select **Duplicate Layer**, and enter the image number as the name in the dialog box. Choose one image as the destination file and click **OK [2-TXT]**.

4.2.1. SCREEN: 68385_screenshot_4.2.1-4.2.2.mp4**:** 00:03-00:05, 00:19-00:23

4.2.2. SCREEN: 68385_screenshot_4.2.1-4.2.2.mp4: 00:31-00:44. **TXT: Repeat for all images**

4.3. Next, use the **Move Tool** to align the images manually and adjust orientation with **Edit, Transform, Rotate,** or **Flip** as needed **[1]**. For finer adjustments, use **Edit, Transform, Distort,** and move the corners individually to match anatomical landmarks **[2]**. Set the blending mode of the top layer to **Overlay** in the **Layers Panel** to facilitate alignment **[3-TXT]**.

4.3.1. SCREEN: 68385_screenshot_4.3.1-4.3.3.mp4: 00:15-00:31

4.3.2. SCREEN: 68385_screenshot_4.3.1-4.3.3.mp4: 02:05-02:25

4.3.3. SCREEN: 68385_screenshot_4.3.1-4.3.3.mp4: 02-45-02:50

TXT: Compare barrel outline and vessels for anatomical accuracy; Save images in PSD or TIFF format

4.4. In each section that displays the barrel cortex, use a stereomicroscope to identify the selected barrel **[1]** and cut it out along with the adjacent one using a razor blade **[2]**.

4.4.1. Shot of the selected barrel and neighboring structure.

4.4.2. Talent cuts the selected section.

4.5. After embedding the section in resin block, collect the ultrathin sections onto Formvar-coated, single-slot nickel grids **[1-TXT]**.

4.5.1. Talent placing ultrathin sections onto the prepared nickel grids. **TXT: Staining: 2% Uranyl acetate, 3 min; 0.03% Lead citrate: 1.5 min**

5. Title: Synapse Analysis and Spine Reconstruction

Demonstrator: Małgorzata Jasińska

5.1. After aligning the TEM images stack as demonstrated earlier, define the analysis area, click **Layer,** followed by **New,** and **Layer**, then click **OK [1]**. Select the **Rectangle Tool**, go to **Layer, Layer Style, Stroke**, **set the stroke color, thickness**, and **position** to **Inside**, then click **OK [2]**. Now, draw a rectangle to define the analysis boundary and select Fill: 0% **[3]**.

5.1.1. SCREEN: 68385_screenshot_5.1.1-5.1.2.mp4: 00:02-00:08

5.1.2. SCREEN: 68385_screenshot_5.1.1-5.1.2.mp4: 00:10-00:30,

5.1.3. SCREEN: 68385_screenshot_5.1.1-5.1.2.mp4: 00:41-00:56

5.2. To add a new layer for annotations, select **Layer, New, Layer**, then click **OK**. Use the **Brush Tool** to mark synapses on this layer **[1]**. Use the **Zoom Tool** and **Hand Tool** to ensure precision while annotating **[2]**.

5.2.1. SCREEN: 68385_screenshot_5.2.1-5.2.2.mp4: 00:41-00:58

5.2.2. SCREEN: 68385_screenshot_5.2.1-5.2.2.mp4: 01:07-01:20

5.3. Now, open the image stack intended for 3D reconstruction. Select the **Crop Tool**, mark the region containing the dendritic spine across the stack, and press **Enter** to crop that region in all visible layers **[1]**. To make only one layer visible at a time, toggle the eye icon in the **Layers Panel**. Click **File, Save As**, name the file, select JPG *(J-P-G)* format, and click **OK** to save each cropped image separately **[2]**.

5.3.1. SCREEN: 68385_screenshot_5.3.1-5.3.2.mp4**:** 00:01-00:09, 00:16-00:19, 00:27-00:36, 00:50-00:55

5.3.2. SCREEN: 68385_screenshot_5.3.1-5.3.2.mp4**:** 01:10-01:30

5.4. Open the 3D reconstruction software. Before uploading, click **Z** on the Navigation Gizmo or press **Numpad 7** *(Num-Pad-Seven)* to align the viewport perspective **[1]**. Next, to upload the images, click **Add, Image, Reference**, then select the first image and double-click to confirm **[2]**.

5.4.1. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 00:02-00:05

5.4.2. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 00:10-00:17.

5.5. To position the final TEM image along the Z axis, click on the image. In the **Object Properties** panel on the right sidebar, enter the appropriate value in the **Location Z** field **[1]**.

5.5.1. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4**:** 01:46-01:52.

5.6. To distribute all images evenly along the Z axis, select all image objects **[1]**. Press **N** to open the **N-panel**, navigate to the **Edit** tab, and click **Dist Z** *(dɪˈstrɪbjut zi)* to automatically space them based on section thickness **[2]**.

5.6.1. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 01:56-02:02

5.6.2. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 02:03-02:13.

5.7. To manually outline the dendritic spine shape on each TEM image using a single curve per image, click **Add, Curve,** and **Circle [1]**. To move the circle, hold **G**, drag the mouse, and then click to confirm the placement. Hold **S** to adjust the size, then click to confirm. Next, scale the object to align with the shape of the dendritic spine **[2]**.

5.7.1. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 02:40-02:46

5.7.2. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 02:51-03:03

5.8. Now, modify the curve shape to fit the spine boundary. Select a control point, press **G**, move the point, and click to confirm**.** Use the **G** or **S** keys as needed to adjust the handle points, refining the curve's direction and intensity **[1].** To add more details, hold Shift and select two neighboring control points, then right-click and choose Subdivide to insert a new point between them **[2].** Once the curve accurately outlines the spine, enter the Layout tab, right-click the BézierCircle object, and choose Duplicate Object without moving the mouse **[3].**

5.8.1. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 03:38-04:10

5.8.2. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 04:50-04:57

5.8.3. SCREEN: 68385_screenshot_5.4.1-5.8.3. mp4: 06:15-06:20

5.9. To connect the edges of the joined object and form a closed mesh, enter the **Modeling** tab **[1]**. Press **2** to switch to **Edge Select** mode. Click **Select, All**, right-click, and choose **Bridge Edge Loops** to link the edge rings together **[2]**.

5.9.1. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 00:01-00:06

5.9.2. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 00:08-00:19

5.10. To fill the gap at the top of the object, deselect all by clicking next to it **[1]**. Hold **Alt** *(Alt)* and click on one edge of the top ledge to select the entire loop. Right-click and choose **Extrude Edges**, drag upward along the Z axis **[2]**. Now, press **S**, move the mouse inward

to shrink the new loop, and click to confirm. With the loop still selected, right-click and choose **New Face from Edges [3]**.

5.10.1. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 00:20-00:25

5.10.2. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 00:25-00:29

5.10.3. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 00:30-00:40

5.11. To smooth the final structure, go to the **Modifiers** tab on the right sidebar. Click **Add Modifier**, navigate to **Generate**, and select **Subdivision Surface [1]**. Set the **Viewport** and **Render** levels to **1** for consistent smoothing **[2]**.

5.11.1. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4**:** 01:19-01:30

5.11.2. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 01:30-01:33

5.12. To assign color to individual structures, select the desired object. Open the **Material** tab on the right sidebar, click **New**, and set the **Base Color [1-TXT]**.

5.12.1. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 01:38-01:3, 02:05-02:09 **TXT: Using the Roughness/Metallic sliders fine tune the appearance**

5.13. Select the **Light** object to adjust scene lighting. Press and hold **G**, move the mouse to reposition the light, and click to confirm its new location **[1]**.

5.13.1. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 02:55-03:10

5.14. To position the camera for rendering, orient the viewport to face the dendritic spine as it should appear in the final output **[1]**. Press **Control, Alt, and Numpad 0** to snap the camera to the current view. Adjust the camera frame by selecting it, holding **G**, and moving it to refine the framing **[2]**.

5.14.1. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 03:14-03:16

5.14.2. SCREEN: 68385_screenshot_5.9.1-5.14.2. mp4: 03:18-03:32

Results

6. Results

6.1. The mouse somatosensory cortex was identifiable even in unstained brain sections **[1],** and the entire barrel field could be reconstructed using digital tools despite the distribution of individual barrels across multiple sections **[2].**

6.1.1. LAB MEDIA: Figure 5A.

6.1.2. LAB MEDIA: Figure 5B.

6.2. Consecutive ultrathin TEM images enabled the reliable classification of synapses into excitatory or inhibitory types based on serial visual evidence **[1]**.

6.2.1. LAB MEDIA: Figure 6. *Video editor: Highlight and zoom the red boxed area*

6.3. Three-dimensional reconstruction of dendritic spines from serial sections allowed for visualization of spine morphology, revealing different shapes such as thin **[1],** mushroom **[2],** stubby spines **[3],** and intermediate **[4].**

6.3.1. LAB MEDIA: Figure 7A–D. *Video editor: Highlight 7A°*

6.3.2. LAB MEDIA: Figure 7A–D. *Video editor: Highlight 7C°*

6.3.3. LAB MEDIA: Figure 7A–D. *Video editor: Highlight 7B°*

6.3.4. LAB MEDIA: Figure 7A–D. *Video editor: Highlight 7D*

6.4. Spine shapes were quantified based on measurements including spine length, neck length, and diameters of the spine head and neck **[1].**

6.4.1. LAB MEDIA: Figure 7E.

1. cerebellum
Pronunciation link:
https://www.merriam-webster.com/dictionary/cerebellum How To Pronounce+7howjsay.com+7Cambridge Dictionary+7Encyclopedia Britannica+12Merriam-Webster+12OpenMD+12
IPA: /ˌser-əˈbel-əm/ Cambridge DictionaryMerriam-Webster
Phonetic Spelling: ser-ə-BEL-əm

2. cacodylate
Pronunciation link:
https://www.merriam-webster.com/medical/cacodylate Wikipedia+12Merriam-Webster+12Merriam-Webster+12
IPA: /ˌkækəˈdɪleɪt/ Merriam-WebsterCollins Dictionary
Phonetic Spelling: KAK-ə-DILL-ayt