FINAL SCRIPT: APPROVED FOR FILMING

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Title: Understanding the Changes in Mitochondrial Morphology Through Dynamic and Three-Dimensional Fluorescent Micrographs

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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? **Yes, all done**

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

Current Protocol Length
Number of Steps: 10
Number of Shots: 36

Introduction

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

1.1. **Sholto de Wet:** The aim of our research is to observe changes in mitochondrial networks and investigate how these mitochondrial networks change in response to cellular conditions **[1]**.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 2 (top row).* NOTE: Use take 4

What technologies are currently used to advance research in your field?

1.2. **Rensu Theart:** We use open-source tools like Fiji and Python, combining existing libraries with custom macros and scripts to automate and scale mitochondrial morphology analysis from complex fluorescence microscopy data **[1]**.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.2.* NOTE: Use take 2, third try

What are the current experimental challenges?

1.3. **Ben Loos:** Achieving reliable quantitative data and metrics that describe the dynamics of mitochondrial fission and fusion, with their localization, remains a challenge. Standardization for such data generation is not commonly available. Therefore, limited knowledge exists on cell-specific fission and fusion frequency and intracellular localization **[1]**.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.2., 4.2.3., 4.2.4.* NOTE: Use take 2

What significant findings have you established in your field?

1.4. **Sholto de Wet:** We added live detection of mitochondrial fission and fusion to the available research metrics. By combining these with mitochondrial structure count, we were able to define a new metric for understanding mitochondrial network dynamics **[1]**.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3A, 3B, 3C.*

How will your findings advance research in your field?

1.5. **Ben Loos:** Our findings allow us to characterize cell-specific fission and fusion parameters and, for the first time, determine whether the mitochondrial system is in equilibrium or shifting. This prevents major misinterpretation of phenotypes in health and disease and provides a clear framework for accurate reporting **[1]**.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. NOTE: Use take 2, second try

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

Protocol

2. Image Preparation, Cell Isolation, and Preprocessing for Mitochondrial Analysis

Demonstrator: Sholto de Wet

2.1. To begin, open the raw file in ImageJ **[1]**. Adjust the color settings to enhance the visibility of the region of interest, but do not set anything **[2]** Duplicate the image according to the number of single cells that need to be analyzed **[3]**. If multiple cells are present in a field of view, navigate to **Analyze**, **Tools**, and use the **synchronize** windows tool and the **freehand drawing tool** to draw a region of interest around a cell of interest **[4]**. Then, select **Edit** and choose **Clear Outside** to isolate the selected cell **[5]**.

2.1.1. WIDE: Talent launching ImageJ and opening a raw image file from a directory. *Videographer: Please record this shot and make sure both the talent launching the software and the computer screen are clearly visible in the frame*. NOTE: split into 2.1.1A (WS) and 2.1.1B (CU)

2.1.2. SCREEN: 68478_screenshot_1.mp4 00:27-00:42.

2.1.3. SCREEN: 68478_screenshot_1.mp4 00:46-01:01.

2.1.4. SCREEN: 68478_screenshot_1.mp4 01:02-01:36.

2.1.5. SCREEN: 68478_screenshot_1.mp4 01:37-02:12.

2.2. Split the red and blue channels from each other **[1]** and save the mitochondrial channel as a .Tiff *(Tif)* file **[2]**.

2.2.1. SCREEN: 68478_screenshot_1.mp4 02:20-02:25.

2.2.2. SCREEN: 68478_screenshot_1.mp4 02:26-02:36.

2.3. To generate a point spread function or PSF *(P-S-F)*, reopen the raw image **[1].** Then, open the **PSF Generator plugin** by selecting **Plugins**, then choosing **PSF Generator,** and selecting the **Born & Wolf 3D Optical Model [2]**. Open the image information of the raw image by selecting **Image**, then choosing **Show Info…** *(Show Information)*, or by pressing **I** *(eye)* on the keyboard **[3]**.

2.3.1. SCREEN: 68478_screenshot_2.mp4 00:10-00:26.

2.3.2. SCREEN: 68478_screenshot_2.mp4 00:27-00:42.

2.3.3. SCREEN: 68478_screenshot_2.mp4 00:42-00:46.

2.4. Scroll to the bottom of the image information window, select the **voxel size and depth** option, and change the Wavelength to 568 nanometers **[1]**. Set the **Pixelsize XY** to 166.1 nanometers, **Z-step** to 200 nanometers, set the Size XYZ to match an image resolution of 512 by 512, and configure the Z-stack to contain 10 Z-slices. Click on **Run [2]**. Save the PSF as a .Tiff file in its own folder **[3]**.

2.4.1. SCREEN: 68478_screenshot_2.mp4 00:46-00:58.

2.4.2. SCREEN: 68478_screenshot_2.mp4 00:59-01:45.

2.4.3. SCREEN: 68478_screenshot_2.mp4 01:46-02:03.

2.5. Navigate to **Plugins**, select **Macros**, then choose **Edit**, followed by **Deconvolution_time_lapse_mine.ijm** *(Deconvolution Time-lapse mine-dot-I-J-M)* to access the deconvolution macro **[1]**. Edit the input and output lines as required and press **Run** to execute the macro **[2]**.

2.5.1. SCREEN: 68478_screenshot_2.mp4 02:10-02:34.

2.5.2. SCREEN: 68478_screenshot_2.mp4 02:43-03:02, 03:11-03:24, 03:38-03:51.

2.6. For image contrast enhancement and blurring, navigate to **Plugins**, select **Macros**, choose **Edit**, and open **Preprocessing.ijm** *(Preprocessing-dot-I-J-M)* to access the preprocessing macro **[1]**. Perform **background subtraction** by setting the **rolling ball radius** to 6 **[2]**. Set the **Sigma Filter Plus** such that the **radius** is 1, the number of **pixels** used is 2, and the **minimum pixel fraction** is 0.2, ensuring the plugin is set to be outlier-aware **[3]**. Adjust the **CLAHE** *(Clay)* settings by configuring the **blocksize** to 64, **histogram bins** to 256, **maximum slope** to 2.5, and **Gamma** to 0.8. Then, click **Run [4]**.

2.6.1. SCREEN: 68478_screenshot_3.mp4 00:05-00:17.

2.6.2. SCREEN: 68478_screenshot_3.mp4 00:30-00:35.

2.6.3. SCREEN: 68478_screenshot_3.mp4 00:36-00:38.

2.6.4. SCREEN: 68478_screenshot_3.mp4 00:38-01:04.

3. Procedure for Image Thresholding

3.1. Open a file of interest that has been modified using the **Preprocessed.ijm** macros in ImageJ **[1]**. Navigate to **Plugins** and select **adaptiveThr** *(adaptive threshold)* **[2]**. Set the **local threshold** to **Weighted Mean** and adjust the pixel block size as required **[3-TXT]**.

To optimize for time, click on **Preview** and adjust the **block size** to clearly include as many mitochondria as possible. Modify the subtract value for each cell to eliminate unnecessary background **[4-TXT].** Take note of the resulting micrograph and sort images into files according to the applied subtract value **[5]**.

3.1.1. SCREEN: 68478_screenshot_4.mp4 00:07-00:30. *Video Editor: Remove 00:17-00:25.*

3.1.2. SCREEN: 68478_screenshot_4.mp4 00:33-00:41.

3.1.3. SCREEN: 68478_screenshot_4.mp4 00:42-00:46. **TXT: The pixel block size should be consistent across cells**

3.1.4. SCREEN: 68478_screenshot_4.mp4 00:46-01:04. **TXT: Take note of the subtract value**

3.1.5. SCREEN: 68478_screenshot_4.mp4 01:24-01:50.

3.2. Now, navigate to **Plugins**, select **Macros**, choose **Edit**, and open **Threshold.ijm** to access the thresholding macro **[1]**. Edit the macro script to define the correct input and output paths, block size, and subtract values **[2]**. Click **Run** to execute the macro **[3]**.

3.2.1. SCREEN: 68478_screenshot_5.mp4 00:05-00:20.

3.2.2. SCREEN: 68478_screenshot_5.mp4 00:28-00:41.

3.2.3. SCREEN: 68478_screenshot_5.mp4 00:42-00:50.

4. Detection of Fission and Fusion Events by the Mitochondrial Event Localizer (MEL) Plugin

4.1. Open up to 10 thresholded micrographs that belong to the same treatment condition **[1]**. Navigate to **Image**, **Stacks**, **Tools**, and select **Concatenate**. Then, press **Ok [2]**. To remove residual small puncta left behind by thresholding, go to **Plugins**, followed by **Integral Image Filters**, and then select **Remove outliers [3]**. Use **Preview** to fine-tune the X and Y sizes to eliminate fragments **[4]**. Save the concatenated file as a TIFF *(Tif-File)* **[5]**.

4.1.1. SCREEN: 68478_screenshot_6.mp4 00:15-00:28.

4.1.2. SCREEN: 68478_screenshot_6.mp4 00:42-01:03.

4.1.3. SCREEN: 68478_screenshot_6.mp4 01:20-01:27.

4.1.4. SCREEN: 68478_screenshot_6.mp4 01:33-01:40, 02:13-02:16.

4.1.5. SCREEN: 68478_screenshot_6.mp4 02:17-02:21, 02:43-02:46, 03:00-03:04.

4.2. Finally, navigate to **Plugins**, **Macros**, **Edit**, **Quicktest_new.ijm** *(Quicktest-New-dot-I-J-M)* **[1]**. Edit the input and output path lines to point to the appropriate directories **[2]**, click **Run [3]**, and visualize the Mitochondrial Event Localizer or MEL *(mel* 'Pronounced like the first bit as in Melbourne'*)* results **[4]**.

4.2.1. SCREEN: 68478_screenshot_6.mp4 03:13-03:21.

4.2.2. SCREEN: 68478_screenshot_6.mp4 03:33-04:04. *Video Editor: Speed up or remove the typing part as needed.*

4.2.3. SCREEN: 68478_screenshot_6.mp4 04:05-04:08.

4.2.4. Talent opening and showing the MEL results. **TXT: MEL detects all fission and fusion events that occur at each timepoint in 3D** NOTE: shot in 4K, use take 2

Videographer: Please record 4.2.4 and make sure the screen is clearly visible in this shot.

Results

5. Results

5.1. Mitochondrial dynamics were tracked over time **[1]**, with red puncta marking fission events **[2]** and green puncta marking fusion events **[3]** in both 3D and 2D views **[4]**.

5.1.1. LAB MEDIA: Figure 2.

5.1.2. LAB MEDIA: Figure 2. *Video Editor: Highlight the red dots.*

5.1.3. LAB MEDIA: Figure 2. *Video Editor: Highlight the green dots*.

5.1.4. LAB MEDIA: Figure 2. *Video Editor: Emphasize the three images in the top row when the VO says "3D" and emphasize the six images in the middle and bottom rows when the VO says "2D views"*.

5.2. Mitochondrial networks showed treatment-specific differences in structure over time **[1]**, with more elongated and interconnected forms in Metformin-treated cells **[2]**, and highly fragmented networks in metformin+CCCP+Baf-treated *(metformin plus C-C-C-P plus Baf treated)* cells **[3]**.

5.2.1. LAB MEDIA: Figure 3A-3C.

5.2.2. LAB MEDIA: Figure 3A-3C. *Video Editor: Highlight B.*

5.2.3. LAB MEDIA: Figure 3A-3C. *Video Editor: Highlight C*.

5.3. The metformin+CCCP+Baf group showed significantly higher fission and fusion activity than the control or metformin-only groups **[1]**, suggesting increased mitochondrial remodeling **[2]**.

5.3.1. LAB MEDIA: Figure 3D. *Video Editor: Highlight the tallest blue and orange bars labeled "Metf+CCCP+Baf".*

5.3.2. LAB MEDIA: Figure 3D.

5.4. This group also had a significantly higher mitochondrial count **[1]**, consistent with enhanced fragmentation **[2]**.

5.4.1. LAB MEDIA: Figure 3E. *Video Editor: Highlight the bar labeled "Metf+CCCP+Baf".*

5.4.2. LAB MEDIA: Figure 3E.

5.5. Mitochondrial volume was significantly reduced in the same group **[1]**, further supporting a shift toward fission **[2]**.

5.5.1. LAB MEDIA: Figure 3F. *Video Editor: Highlight the bar labeled "Metf+CCCP+Baf".*

5.5.2. LAB MEDIA: Figure 3F.

5.6. However, when normalized to mitochondrial number, the metformin-only group exhibited the highest relative dynamic activity **[1]**, suggesting that metformin alone promotes a more active and efficient remodeling network, while co-treatment drives extensive but less efficient structural turnover **[2]**.

5.6.1. LAB MEDIA: Figure 3G. *Video Editor: Highlight the bar labeled "Metf".*

5.6.2. LAB MEDIA: Figure 3G

Pronunciation Guide:

1. mitochondrial

Pronunciation link: https://www.merriam-webster.com/dictionary/mitochondrion
explore.albumentations.ai+15merriam-webster.com+15howtopronounce.com+15
IPA (American): /ˌmaɪtəˈkɑn.dri.əl/
Phonetic Spelling: my-tuh-KON-dree-uhl

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2. mitochondrion

Pronunciation link: https://www.merriam-webster.com/dictionary/mitochondrion
IPA (American): /ˌmaɪtəˈkɑn.dri.ən/
Phonetic Spelling: my-tuh-KON-dree-uhn

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3. voxel

(Not found on Merriam, but a common term in imaging)
Pronunciation link: No confirmed link found
IPA (American): /ˈvɑk.səl/
Phonetic Spelling: VOK-suhl

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4. deconvolution

(No Merriam page found)
Pronunciation link: No confirmed link found
IPA (American): /ˌdiːˌkɒn.vəˈluː.ʃən/
Phonetic Spelling: dee-kon-vuh-LOO-shun

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5. concatenated

Pronunciation link: No confirmed link found
IPA (American): /kəˈnæk.təˌneɪ.tɪd/
Phonetic Spelling: kuh-NAK-tuh-nay-tid

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6. puncta

(Latin plural for points)
Pronunciation link: No confirmed link found
IPA (American): /ˈpʌŋk.tə/
Phonetic Spelling: PUNK-tuh

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7. fission

Pronunciation link: No confirmed link found
IPA (American): /ˈfɪʒ.ən/
Phonetic Spelling: FIZH-uhn

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8. fusion

Pronunciation link: No confirmed link found
IPA (American): /ˈfjuz.ən/
Phonetic Spelling: FYOO-zhun

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9. metformin

(Generic drug name—may not appear in Merriam)
Pronunciation link: No confirmed link found
IPA (American): /mɛtˈfɔr.mɪn/
Phonetic Spelling: met-FOR-min
