

Submission ID #: 70583

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Project Page Link: https://review.jove.com/files_upload.php?src=21527863

Title: Focus Stacking Protocol for High-resolution Insect Photography

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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. Interview statements: Which interview statement filming option is the most appropriate for your group? **Please select one.**



Interviewees self-record interview statements. JoVE can provide support for this option.

3. Proposed interview filming date: Please indicate the proposed date that your group will self-film interviews:

When you are ready to submit your video files, please contact our Content Manager, [Utkarsh Khare](#).

Current Protocol Length

Number of Steps: 17

Number of Shots: 25

Interviews

NOTE to VO: Please record the interview statements

INTRODUCTION:

REQUIRED: Summarize your research focus and the main questions it aims to answer.

- 1.1. This research explores how human activities, deforestation, climate change, and land-use changes affect mosquito dynamics, biodiversity, ecosystem health, and pathogen transmission.

1.1.1. [3.1.1.](#)

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. Current methods struggle with insect identification and large dataset analysis. This image-stacking protocol enhances diagnostic image quality, improving the reliability of identification.

1.2.1. [4.3.1](#)

CONCLUSION

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.3. The greatest challenge while performing this protocol was eliminating seismic vibrations and airflow to capture extremely high-resolution images of very small insects.

1.3.1. [2.1.2](#)

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.4. Future studies could combine these images with machine learning to discover new morphological traits and improve species identification.

- 1.4.1. [4.5.1](#)

Protocol

2. Setting up the Imaging Workstation

Demonstrator: Nita Soun

- 2.1. To begin, place a vibration-damping table on a stable surface [1]. Position a camera mounting rail at the center of the table [2] and secure the digital camera body to the rail with a rigid plate adapter [3].
 - 2.1.1. WIDE: Talent placing a vibration-damping table on a stable surface.
 - 2.1.2. Talent positioning a camera mounting rail at the center of the table.
 - 2.1.3. Talent fixing the digital camera body onto the rail with a rigid plate adapter.
- 2.2. Attach the selected microscope objective from 5X (Five-X) to 20X or a macro lens via the appropriate tube adapter [1].
 - 2.2.1. Talent attaching the selected microscope objective or macro lens using the appropriate tube adapter.
- 2.3. Then, install two rear and two frontal light sources equipped with diffusers to create uniform illumination [1].
 - 2.3.1. Shot of the installed rear and frontal light sources equipped with diffusers.
- 2.4. Mount the subject support consisting of an XYZ (X-Y-Z) stage or rotary holder directly beneath the lens axis [1].
 - 2.4.1. Talent mounting the subject support directly beneath the lens axis.
- 2.5. Ensure that the optical axis is perpendicular to the specimen plane [1].
 - 2.5.1. Talent checking and aligning the optical axis perpendicular to the specimen plane.
- 2.6. Adjust the horizontal and vertical level using the stage micrometers until reflections are symmetrical [1].
 - 2.6.1. Talent adjusting the horizontal and vertical level with the stage micrometers.
- 2.7. Confirm that illumination is homogeneous across the field [1] and realign the diffusers as needed, followed by color calibration [2].
 - 2.7.1. Talent checking the illumination across the field.
 - 2.7.2. Talent realigning the diffusers to achieve homogeneous illumination.

3. Specimen Mounting and Focusing

- 3.1. Fix dried or ethanol-preserved insects on a soft mounting base such as modeling clay

[1] and orient the specimen according to the desired view [2].

3.1.1. LAB MEDIA: 8.Mov: 01:16-01:30

3.1.2. LAB MEDIA: 11.Mov: 00:05-00:19

3.2. Position the support centered under the optical axis [1].

3.2.1. LAB MEDIA: 12.Mov: 00:00-00:12.

3.3. Define the beginning and end of the focal range with the nearest and farthest sharp plane [1].

3.3.1. LAB MEDIA:24.Mov 01:00-01:21

3.4. Program incremental movement between 0.2 micrometers and 2 micrometers depending on magnification [1]. Then, synchronize the capture trigger so that illumination fires simultaneously with each image exposure [2].

3.4.1. LAB MEDIA: 24.Mov 00:28-00:51

3.4.2. LAB MEDIA: 24. Mov: 00:10-00:26

3.5. Capture sequences of 50 to 2000 images according to specimen size and depth [1].

3.5.1. LAB MEDIA: 25.Mov: 00:00-00:09

4. Image Processing

4.1. Transfer the files to a local computer immediately after acquisition [1]. Group each stack into a unique folder using incremental numbering [2].

4.1.1. LAB MEDIA: 26.Mov: 00:10-00:23

4.1.2. LAB MEDIA: 27.Mov: 00:00-00:09

4.2. Apply lens and color correction profiles [1], then export the images as 16-bit TIFF files [2].

4.2.1. LAB MEDIA: 27.Mov: 00:30-00:36

4.2.2. LAB MEDIA: 29.Mov: 00:11-00:16.

4.3. Open the TIFF stack in focus stacking software [1].

4.3.1. LAB MEDIA: 29.Mov: 0:17-0:20

4.4. Choose a depth map algorithm for the first composite [1].

4.4.1. SCREEN: 4.4.1.-depth-map-algorithm.mp4: 00:40-00:45.

4.5. Then, open the composite in an image editing program [1]. Remove background artifacts and dust using selection and healing tools [2].

4.5.1. LAB MEDIA: 38.Mov: 00:00-00:10

4.5.2. LAB MEDIA: 38.Mov: 00:51-01:10

Results

5. Results

- 5.1. The focus stacking system produced fully sharp, high contrast composites across magnifications from 5X to 20X [1]. Calculated pixel scales ranged from 0.76 micrometers at 5X magnification [2] to 0.19 micrometers at 20X magnification in object space, confirming adequate sampling for sub-micron structural details [3].
 - 5.1.1. LAB MEDIA: Figure 2 and 3
 - 5.1.2. LAB MEDIA: Table 1. *Video editor: Highlight the value 0.752 in row 5× under “Scale per Pixel (Object Plane, micrometer)”.*
 - 5.1.3. LAB MEDIA: Table 1. *Video editor: Highlight the value 0.188 in row 20× under “Scale per Pixel (Object Plane, micrometer)”.*
- 5.2. The effective resolving power was approximately 4 micrometers [1], matching the diffraction limit of the employed objectives [2].
 - 5.2.1. LAB MEDIA: Table 2. *Video editor: Highlight the Airy disk diameter values near 4 micrometers in the “Airy disk diameter (Object plane, micrometer)” column.*
 - 5.2.2. LAB MEDIA: Table 2. *Video editor: Highlight the entries “Diffraction” in the “Limiting factor” column.*
- 5.3. Expected depth of field values ranged from 5.05 micrometers [1] to approximately 0.1 micrometers [2].
 - 5.3.1. LAB MEDIA: Table 2. *Video editor: Highlight the value 5.05 in the “Depth of field (micrometer)” column.*
 - 5.3.2. LAB MEDIA: Table 2. *Video editor: Highlight the value approximately 0.1 in the “Depth of field (micrometer)” column.*

1. Vibration-damping
Pronunciation link: No confirmed link found
IPA: /vaɪˈbreɪʃən ˈdæmpɪŋ/
Phonetic Spelling: vy-BRAY-shuhn DAM-ping
2. Microscope
Pronunciation link: <https://www.merriam-webster.com/dictionary/microscope>
IPA: /ˈmaɪkrəˌskoʊp/
Phonetic Spelling: MY-kruh-skohp
3. Diffuser
Pronunciation link: <https://www.merriam-webster.com/dictionary/diffuser>
IPA: /dɪˈfjuːzər/
Phonetic Spelling: dih-FYOO-zer
4. Illumination
Pronunciation link: <https://www.merriam-webster.com/dictionary/illumination>
IPA: /ɪˌluːməˈneɪʃən/
Phonetic Spelling: ih-loo-muh-NAY-shuhn
5. Micrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪˈkrɑːmətər/
Phonetic Spelling: my-KROM-uh-ter
6. Homogeneous
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogeneous>
IPA: /ˌhoʊməˈdʒiːniəs/
Phonetic Spelling: hoh-muh-JEE-nee-uhs
7. Calibration
Pronunciation link: <https://www.merriam-webster.com/dictionary/calibration>
IPA: /ˌkælɪˈbreɪʃən/
Phonetic Spelling: kal-ih-BRAY-shuhn
8. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /ˈεθəˌnɔːl/
Phonetic Spelling: ETH-uh-nawl
9. Specimen
Pronunciation link: <https://www.merriam-webster.com/dictionary/specimen>
IPA: /ˈspesəmən/
Phonetic Spelling: SPESS-uh-muhn
10. Focal
Pronunciation link: <https://www.merriam-webster.com/dictionary/focal>
IPA: /ˈfoʊkəl/
Phonetic Spelling: FOH-kuhl
11. Synchronize
Pronunciation link: <https://www.merriam-webster.com/dictionary/synchronize>
IPA: /ˈsɪŋkrəˌnaɪz/
Phonetic Spelling: SING-kruh-nyze
12. Acquisition
Pronunciation link: <https://www.merriam-webster.com/dictionary/acquisition>

IPA: /,ækwə'zɪʃən/

Phonetic Spelling: ak·wuh·ZISH·uhn

13. Incremental

Pronunciation link: <https://www.merriam-webster.com/dictionary/incremental>

IPA: /,ɪŋkrə'mentəl/

Phonetic Spelling: in·kruh·MEN·tuhl

14. Algorithm

Pronunciation link: <https://www.merriam-webster.com/dictionary/algorithm>

IPA: /'ælgə,rɪðəm/

Phonetic Spelling: AL·guh·rith·uhm

15. Composite

Pronunciation link: <https://www.merriam-webster.com/dictionary/composite>

IPA: /kəm'pəːzət/

Phonetic Spelling: kuhm·POZ·it

16. Magnification

Pronunciation link: <https://www.merriam-webster.com/dictionary/magnification>

IPA: /,mægnəfə'keɪʃən/

Phonetic Spelling: mag·nuh·fuh·KAY·shuhn

17. Diffraction

Pronunciation link: <https://www.merriam-webster.com/dictionary/diffraction>

IPA: /dɪ'frækʃən/

Phonetic Spelling: dih·FRAK·shuhn

18. Airy disk

Pronunciation link: No confirmed link found

IPA: /'ɛəri dɪsk/

Phonetic Spelling: AIR·ee disk

19. Resolving power

Pronunciation link: No confirmed link found

IPA: /rɪ'zɔːlvɪŋ 'paʊər/

Phonetic Spelling: rih·ZOLV·ing POW·er

20. Depth of field

Pronunciation link: No confirmed link found

IPA: /dɛpθ əv fiːld/

Phonetic Spelling: depth·uhv·FEELD