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Project Page Link: <https://review.jove.com/account/file-uploader?src=21737703>

Title: Plate-based Imaging Assay for Quantifying Photoperiodic Responses in *Neurospora crassa* Using Protoperithecia Development

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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? **Yes, all done**

- 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.**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes.**

Current Protocol Length

Number of Steps: 29

Number of Shots: 43

Interviews

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

INTRODUCTION:

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

- 1.1. **Aleece Siner:** Our research focuses on understanding fungal photoperiodic regulation by measuring protoperithecia formation under controlled light-dark cycles.

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

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

- 1.2. **Kwangwon Lee:** Existing photoperiod assays lack standardized methods for fungal studies, while this protocol provides reproducible protoperithecia-based photoperiod analysis.

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

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Aleece Siner:** This protocol enables researchers to quantify protoperithecia development as a measure of fungal responses to changing day lengths.

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

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

- 1.4. **Aleece Siner:** The greatest challenge in this protocol is maintaining consistent light intensity, environmental conditions, and plate hydration throughout the experiment.

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

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

1.5. **Kwangwon Lee:** Future studies can build on this work by investigating genetic pathways underlying fungal photoperiodism and developing automated protoperithecia quantification methods.

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

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

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

~~What problem in your field of research does your JoVE article address?~~

- 1.6. **Aleece Siner, Graduate Student Researcher, Rutgers University:** (authors will present their testimonial statements live)

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

~~How does the video format of your article help you to share your work with the broader scientific community?~~

- 1.7. **Aleece Siner, Graduate Student Researcher, Rutgers University:** (authors will present their testimonial statements live)

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

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

Videographer's NOTE: Reshoots - A couple shots got reshot toward the end, when the client realized she had used the wrong Petri dishes initially. So just look out for that.

4.4.x - I'm afraid we missed shots 4.4.1, 4.4.2, and 4.4.3. I mentioned them at the end of the shoot, and the client believed we had already shot them. That said, we shot extra shots 4.x.1 and 4.x.2, where she presented the clean lid and agar surface, respectively. I think that may have been what she was thinking of, and hopefully will be a suitable alternative. It seems like the only thing we're really missing is applying ethanol to the paper towel (4.4.1).

NOTE: SCREEN timestamps for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Sri Lakshmi Priya) for queries regarding lab media.

2. Preparation of Media and Inoculation of Plates

Demonstrator: Aleece Siner

- 2.1. To begin, gather the required reagents [1]. Prepare the synthetic crossing or SC (S-C) medium [2]. Autoclave the medium [3] and allow it to cool to 50 to 55 degrees Celsius until it reaches a pourable state [4].

- 2.1.1. WIDE: Talent arranging the bottles on a workbench.

- 2.1.2. TEXT ON A PLAIN BACKGROUND:

Synthetic Crossing Medium:

0.2% sucrose

5 µg/L biotin

1.5% agar

Westergaard's salts (Adjusted to pH 6.5 with 1 N NaOH)

- 2.1.3. Talent placing the flask containing the prepared medium into the autoclave.

- 2.1.4. Shot of flask placed on the laboratory bench for cooling.

- 2.2. Using a disposable pipette, pipette 10 milliliters of molten medium into 60-millimeter Petri dishes [1]. Allow the plates to solidify at room temperature for 30 to 45 minutes [2].

- 2.2.1. Talent dispensing molten medium into 60 millimeter Petri dishes using a disposable pipette.

- 2.2.2. Shot of the poured plates resting on the bench for solidification.
- 2.3. Store the plates upside down at 4 degrees Celsius for up to 4 weeks [1].
 - 2.3.1. Talent placing the solidified Petri dishes upside down into a refrigerator.
- 2.4. Grow each fungal strain in small glass test tubes containing complete or minimal medium prepared according to laboratory manual instructions [1].
 - 2.4.1. Shot of fungal strains in a small glass test tube containing growth medium.
- 2.5. Incubate the test tubes in constant darkness at 30 degrees Celsius [1] until visible mycelial coverage forms along the tube surface [2].
 - 2.5.1. Talent placing the wrapped test tube into an incubator.
 - 2.5.2. Close-up shot of mycelial coverage along the tube surface.
- 2.6. Using an inoculation loop, apply approximately 1 to 2 microliters of concentrated conidial suspension at the center of the plate to standardize inoculation across plates and ensure uniform growth [1]. Observe the conidia appearing as powdery, light-colored spores covering the colony surface [2].
 - 2.6.1. Talent applying concentrated conidial suspension onto the center of the plate using an inoculation loop.
 - 2.6.2. Close shot of powdery, light-colored conidia on the colony surface.
- 2.7. Close the plates securely to prevent contamination while allowing gas exchange [1].
 - 2.7.1. Talent closing the inoculated Petri dish securely.
- 2.8. Label each plate with the strain name, date, and photoperiod condition [1].
 - 2.8.1. Talent labeling the Petri dish with strain name, date, and photoperiod condition.

3. Photoperiod Treatment Setup

- 3.1. Set the growth chamber temperature to 25 degrees Celsius [1] and humidity to 50% to prevent plate drying and calibrate it according to the manufacturer's instructions [2].
 - 3.1.1. Close-up of talent adjusting the temperature settings of the growth chamber to 25 degrees Celsius.
 - 3.1.2. Talent adjusting the humidity controls on the growth chamber.

NOTE: 3.2 was not filmed. The removed step was concisely added in the previous step.

- ~~3.2. Program the photoperiod cycles to 12 hours light and 12 hours dark for equinox conditions [1] and 8 hours light and 16 hours dark for short day conditions [2].~~

~~3.2.1. SCREEN: Show the photoperiod settings being set to 12 hour light and 12 hour dark for equinox conditions.~~

~~3.2.2. SCREEN: Show the photoperiod settings being set to 8 hour light and 16 hour dark for short day conditions.~~

3.3. Using a calibrated light meter, set the light intensity to greater than 1000 lux at plate level [1]. Avoid light intensities that increase the chamber temperature above 25 degrees Celsius [2].

3.3.1. Shot of light intensity being set to >1000 lux at plate level using a calibrated light meter.

3.3.2. Close shot of the chamber temperature displaying 25 degrees Celsius.

3.4. Arrange the plates evenly on the chamber shelves to ensure uniform light exposure [1].

3.4.1. Talent evenly spacing plates across the shelves inside the growth chamber.

3.5. Place a data logger inside the chamber to continuously monitor temperature, humidity, and verify stable environmental conditions [1].

3.5.1. Talent placing a data logger inside the growth chamber.

3.6. Incubate the plates under the programmed photoperiod for 7 days without disturbance [1].

3.6.1. Shot of the plates inside the growth chamber.

4. Monitoring and Preparation of Plates for Imaging

4.1. Remove the plates from the growth chamber after 7 days of incubation [1].

4.1.1. Talent removing the plates from the growth chamber.

4.2. Inspect the plates for conidial growth and protoperithecia formation [1].

4.2.1. Close shot of the conidial growth and protoperithecia formation in the plate.

4.3. Using torn paper towels, remove surface conidia from the plate lid [1] and from the agar surface [2].

4.3.1. Talent wiping conidia from the surface of the plate lid using torn paper towels.

4.3.2. Talent gently removing conidia from the agar surface using torn paper towels.

4.4. After moistening a paper towel with 1 to 2 milliliters of 70% ethanol, gently dab the plate surface 3 to 5 times to remove conidia [1]. Allow the plates to air-dry for 1 to 3 minutes before imaging [2]. NOTE: For removed shot, VO is edited and combined with next step

4.4.1. Talent applying 70% ethanol onto a paper towel. NOTE: Not filmed.

- 4.4.2. Talent gently dabbing the plate surface with the moistened paper towel.
- 4.4.3. Shot of the cleaned plates placed on the laboratory bench.

5. Imaging and Quantification of Protozoa

- 5.1. Set up a microscope with consistent illumination and a magnification of 40x [1].
 - 5.1.1. Close-up of the talent adjusting the microscope illumination controls and setting the microscope to 40x magnification.
- 5.2. Ensure the plate surface is dry and free of debris [1].
 - 5.2.1. Close shot of the dry and clean agar plate surface.
- 5.3. Image four evenly spaced quadrants at the plate perimeter using consistent magnification [1].
 - 5.3.1. LAB MEDIA: JOVE_IMAGE_EXAMPLE-(5.3.1).tiff
- 5.4. Label each image with the strain name, photoperiod condition, plate number, and quadrant position [1].
 - 5.4.1. SCREEN: 5.4-and-5.5-combined.mp4: 00:00-00:40 NOTE: 5.4.1 and 5.5.1 were combined in one shot. Show the SC only once
- 5.5. Save the images in a TIFF (*tiff*) format for analysis [1].
 - 5.5.1. SCREEN: 5.4-and-5.5-combined.mp4: 00:00-00:40
- 5.6. Open each image in an image processing package [1].
 - 5.6.1. SCREEN: 5.6.mp4: 00:00-00:12
- 5.7. Use the **Cell Counter** plugin to manually count the protozoa [1]. Exclude bubbles, edge artifacts, and ambiguous structures, and count distinguishable clustered protozoa as individual units [2].
 - 5.7.1. SCREEN: 5.7.1.mp4: 00:00-00:13
 - 5.7.2. SCREEN: 5.7.2.mp4: 00:00-00:20
- 5.8. After recording the counts for each quadrant, and calculate the average count per plate [1]. NOTE: For removed shot, VO is edited and combined with next step
 - 5.8.1. SCREEN: Talent entering the count values for each quadrant into a data-sheet. NOTE: Not filmed.
 - 5.8.2. SCREEN: 5.8.mp4: 00:00-00:14
- 5.9. Import the quantified data into statistical analysis software for analysis [1].
 - 5.9.1. SCREEN: 5.9.mp4: 00:00-00:23

Results

6. Results

- 6.1. Plates grown under equinox-like photoperiods exhibited dense protoperithecia formation [1], whereas short-day conditions produced fewer structures [2].
 - 6.1.1. LAB MEDIA: Figure 2A. *Video editor: Emphasize the lower-left counted image labeled "EQ"*
 - 6.1.2. LAB MEDIA: Figure 2A. *Video editor: Emphasize the lower-right counted image labeled "SDP"*.
- 6.2. Quantification using an image-processing package revealed higher average protoperithecia counts under 12-hour light and 12-hour dark cycles [1] compared to 8-hour light and 16-hour dark conditions [2].
 - 6.2.1. LAB MEDIA: Figure 2A, B. *Video editor: In figure 2B, emphasize the bar labelled EQ.*
 - 6.2.2. LAB MEDIA: Figure 2A, B. *Video editor: In figure 2B, emphasize the bar labelled SDP.*

Pronunciation guides:

- 1. Autoclave
Pronunciation link: <https://www.merriam-webster.com/dictionary/autoclave>
IPA: /'ɔːtəˌkleɪv/
Phonetic Spelling: aw·tuh·klayv
- 2. Celsius
Pronunciation link: <https://www.merriam-webster.com/dictionary/Celsius>
IPA: /'selsiəs/
Phonetic Spelling: sel·see·uhs
- 3. Sucrose
Pronunciation link: <https://www.merriam-webster.com/dictionary/sucrose>
IPA: /'suːˌkroʊs/
Phonetic Spelling: soo·krohs
- 4. Biotin
Pronunciation link: <https://www.merriam-webster.com/dictionary/biotin>
IPA: /'baɪətɪn/
Phonetic Spelling: bye·uh·tin
- 5. Agar
Pronunciation link: <https://www.merriam-webster.com/dictionary/agar>

- IPA: /'eɪgɑːr/, /'ɑːgɑːr/
Phonetic Spelling: ay-gahr
6. Westergaard
Pronunciation link: No confirmed link found
IPA: /'wɛstərˌgɑːrd/
Phonetic Spelling: wes-ter-gard
7. Sodium Hydroxide (NaOH)
Pronunciation link: <https://www.merriam-webster.com/dictionary/sodium%20hydroxide>
IPA: /'soʊdiəm hæˈdrɑːksaɪd/
Phonetic Spelling: soh-dee-uhm hy-drok-side
8. Petri
Pronunciation link: <https://www.merriam-webster.com/dictionary/Petri%20dish>
IPA: /'piːtri/
Phonetic Spelling: pee-tree
9. Fungal
Pronunciation link: <https://www.merriam-webster.com/dictionary/fungal>
IPA: /'fʌŋɡəl/
Phonetic Spelling: fung-guhl
10. Mycelial
Pronunciation link: <https://www.merriam-webster.com/dictionary/mycelium>
IPA: /maɪ'siːliəl/
Phonetic Spelling: my-see-lee-uhl
11. Inoculation
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculation>
IPA: /ɪˌnɑːkjəˈleɪʃən/
Phonetic Spelling: ih-naak-yuh-lay-shuhn
12. Conidial
Pronunciation link: <https://www.howtopronounce.com/conidial>
IPA: /kəˈnɪdiəl/
Phonetic Spelling: kuh-nid-ee-uhl
13. Conidia
Pronunciation link: <https://www.howtopronounce.com/conidia>
IPA: /kəˈnɪdiə/
Phonetic Spelling: kuh-nid-ee-uh
14. Photoperiod
Pronunciation link: <https://www.merriam-webster.com/dictionary/photoperiod>
IPA: /'foʊtəʊˌpɪriəd/
Phonetic Spelling: foe-toh-peer-ee-uhd
15. Protoperithecia
Pronunciation link: <https://www.howtopronounce.com/protoperithecia>
IPA: /ˌproʊtəʊˌpɛrɪˈθiːʃiə/
Phonetic Spelling: proh-toh-peh-ruh-thee-shee-uh

16. Equinox
Pronunciation link: <https://www.merriam-webster.com/dictionary/equinox>
IPA: /'i:kwə,nɑ:ks/
Phonetic Spelling: ee·kwuh·naaks
17. Calibrated
Pronunciation link: <https://www.merriam-webster.com/dictionary/calibrated>
IPA: /'kælə,breɪtɪd/
Phonetic Spelling: kal·uh·bray·tuhd
18. Magnification
Pronunciation link: <https://www.merriam-webster.com/dictionary/magnification>
IPA: /,mæɡnəfə'keɪʃən/
Phonetic Spelling: mag·nuh·fuh·kay·shuhn
19. Quadrant
Pronunciation link: <https://www.merriam-webster.com/dictionary/quadrant>
IPA: /'kwɑ:drənt/
Phonetic Spelling: kwah·druhnt
20. TIFF
Pronunciation link: <https://www.merriam-webster.com/dictionary/TIFF>
IPA: /tɪf/
Phonetic Spelling: tif
21. Quantification
Pronunciation link: <https://www.merriam-webster.com/dictionary/quantification>
IPA: /,kwɑ:ntəfə'keɪʃən/
Phonetic Spelling: kwahn·tuh·fuh·kay·shuhn
22. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /'maɪkroʊ,lɪ:tər/
Phonetic Spelling: my·kroh·lee·ter
23. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /'eθə,nɔ:l/
Phonetic Spelling: eth·uh·nawl