

Submission ID #: 71222

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Title: A Fluorescence-Based Method to Visualize Lipid Droplet and Organelle Dynamics during Mitosis in Hepatoma Cells

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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**
- 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. **No**

Current Protocol Length

Number of Steps: 13

Number of Shots: 33 (7 SC)

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. **Tomohiko Makiyama:** Our research focuses on how lipid droplets behave during cell division and their potential roles in mitotic regulation.

- 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. **Tomohiko Makiyama:** Existing methods inadequately track lipid droplet dynamics during mitosis; this protocol offers a simple fluorescence-based imaging approach.

- 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. **Tomohiko Makiyama:** This protocol enables visualization and quantitative analysis of lipid droplet distribution during different stages of mitosis.

- 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. **Tomohiko Makiyama:** Preserving mitotic cell morphology during synchronization, fixation, and staining is critical for reliable imaging results.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Tomohiko Makiyama:** This method can be combined with organelle co-staining and quantitative image analysis for broader cellular investigations.

- 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.

Protocol

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

2. G2/M Synchronization in Hepatoma Cells

Demonstrator: Tomohiko Makiyama

- 2.1. To begin, remove the three 35-millimeter culture dishes, each containing glass coverslips seeded with 2×10^5 HuH7 (*Huh-seven*) cells from the incubator [1]. Wash the cells two times with 1 milliliter PBS [2]. Label the culture dishes as untreated asynchronous control, oleic acid-treated control, and RO-3306 (*R-O-three-three-zero-six*)-treatment [3].
 - 2.1.1. WIDE: Talent removing the three 35 mm culture dishes with coverslips from the incubator. ***Videographer:** Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent washing the cells with phosphate-buffered saline.
 - 2.1.3. Talent labelling the 35-millimeter dishes with corresponding treatment group names
- 2.2. For the untreated asynchronous control, add 2 milliliters of standard DMEM [1]. For the oleic acid- control, add 2 milliliters of DMEM containing 0.5 millimolar oleic acid-BSA (*B-S-A*) conjugate [2], and for the treatment group, add fresh medium containing 9 micromolar RO-3306 and 0.5 millimolar oleic acid-BSA to synchronize the cells at G2/M (*G-two-M*) phase [3].
 - 2.2.1. Talent adding standard DMEM to the untreated asynchronous group.
 - 2.2.2. Talent adding 0.5mM oleic acid-BSA to the oleic acid control group.
 - 2.2.3. Talent adding 9 μ M RO-3306 and 0.5 mM oleic acid-BSA to the treatment group.
- 2.3. After incubating the cells for 20 hours under standard culture conditions, wash the cells with 1 milliliter of PBS three to five times [1]. Then, wash the cells once with 2 milliliters of fresh culture medium [2].
 - 2.3.1. Talent washing the cells with 1X PBS.
 - 2.3.2. Talent washing the cells with fresh culture medium.

3. Preparation of Mitotic Cells and Staining

- 3.1. To facilitate cell entry into mitotic phase, add 2 milliliters of fresh medium to the cells

[1] and incubate for 1 hour at 37 degrees Celsius in a 5 percent carbon dioxide incubator [2]. Then, discard the media [3]. To fix the cells, incubate them in 1 milliliter of 4 percent paraformaldehyde in PBS for 10 minutes at room temperature [4].

3.1.1. Talent adding 2 milliliters of fresh media to the cells.

3.1.2. Talent placing the culture dish in the incubator. *Videographer: Please capture the CO₂ incubator's digital display.*

3.1.3. Talent discarding the media.

3.1.4. Talent adding paraformaldehyde solution to the cells for fixation.

3.2. Permeabilize the fixed cells with 0.1 percent Triton X-100 in PBS for 10 minutes at room temperature [1]. After removing the Triton X-100 solution, wash once with PBS [2]. Block the cells with 1 percent BSA in PBS at room temperature for 1 hour [3].

3.2.1. Talent adding Triton X-100 solution to the fixed cells.

3.2.2. Talent washing the cells with PBS.

3.2.3. Talent adding BSA blocking solution to the cells.

3.3. Wash the cells two times with PBS [1]. Transfer the coverslip containing fixed cells to a humid chamber lined with PBS-moistened paper to prevent the cells from drying [2]. Add the mouse anti-alpha tubulin primary antibody diluted 1 to 500 in PBS and incubate the cells for 1 hour at room temperature [3].

3.3.1. Talent washing the cells with PBS. *Videographer: Obtain multiple reusable takes of 3.3.1, as these will be used later in the protocol.*

3.3.2. Talent transferring the coverslip to a humid chamber.

3.3.3. Talent adding primary antibody solution to the coverslip in the humid chamber.

3.4. After washing the cells two times with PBS, add Alexa Fluor-conjugated anti-mouse IgG (I-G-G) secondary antibody diluted 1 in 500 in PBS and incubate the cells for 1 hour at room temperature [1].

3.4.1. Talent adding secondary antibody solution to the coverslip in the humid chamber.

3.5. Wash two times with PBS [1], then add 150 microliters of a neutral lipid fluorescent dye, BODIPY 493/503 (BOH-dee-pee four-ninety-three-five-oh-three) diluted 1 in 1000 in PBS for 10 minutes at room temperature [2].

3.5.1. Talent washing the sample with PBS

3.5.2. Talent adding neutral lipid fluorescent dye to the coverslip.

3.6. Following two PBS washes, stain the nuclei with DAPI (DAH-pee) diluted 1 in 1000 in PBS for 5 minutes [1].

3.6.1. Talent adding the DAPI solution to the coverslip.

3.7. Repeat washing the cells two times with PBS [1] and mount the coverslips on glass slides using an antifade mounting medium to preserve fluorescence during imaging [2]. Seal the edges of the coverslips with clear nail polish to prevent drying and movement during imaging [3].

3.7.1. Talent washing the sample with PBS

3.7.2. Talent mounting the coverslip onto a glass slide using antifade mounting medium.

3.7.3. Talent sealing the edges of the coverslip with clear nail polish.

4. Imaging and Analysis

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

4.1. Use widefield microscopy [1] to identify and image representative interphase and mitotic cells [2]. Next, use appropriate filter sets to visualize lipid droplets and nuclear morphology [3].

4.1.1. Talent operating the widefield fluorescence microscope to screen multiple cells.

4.1.2. SCREEN: Show the capturing of representative interphase and mitotic cells.
NOTE: SCs are filmed by the videographer

4.1.3. SCREEN: Show lipid droplets and nuclei visualized under appropriate fluorescence filter settings.

4.2. For high-resolution validation, acquire fluorescence images using confocal microscopy [1]. Use appropriate excitation and emission filter settings [2] to detect fluorescence signals from neutral lipids and the DNA-binding dyes [3].

4.2.1. Talent operating the confocal microscope equipped with a high numerical aperture objective lens.

4.2.2. SCREEN: Show confocal microscope interface with excitation and emission filter configurations.

4.2.3. SCREEN: Show acquired fluorescence images on the confocal microscope interface.

4.3. After opening the acquired images in **ImageJ** software, use the ROI (*R-O-I*) selection tool to manually draw the outline for the nucleus [1]. Select the autosaved file, then click **Rename** to change the selected image's name [2]. Next, draw the lipid droplet outlines and rename the files [3]. Select the **Show All** option to display all the drawn outlines [4]. Next, click on **Measure** to obtain the mean size and area of the selected organelles [5].

4.3.1. SCREEN: Jove-Recording-02.mp4: 0:00–0:06

- 4.3.2. SCREEN: Jove-Recording-02.mp4: 0:07–0:10
- 4.3.3. SCREEN: Jove-Recording-02.mp4: 0:11–0:22
- 4.3.4. SCREEN: Jove-Recording-02.mp4: 0:35–0:38
- 4.3.5. SCREEN: Jove-Recording-02.mp4: 0:39–0:44

Results

5. Results

- 5.1. Over 50 percent of cells reached G2/M synchronization after 20 hours of RO-3306 treatment, followed by 1 hour in fresh medium [1]. Before RO-3306 removal, HuH7 cells showed an epithelial-like morphology [2]. One hour after washout, mitotic cells became rounded and partially detached from the culture dish [3].
 - 5.1.1. LAB MEDIA: Figure 1. *Video editor: Please emphasize the Y-axis value 50 and the black solid fill portion of the bar corresponding to RO-3306 on X-axis and the figure legend showing G2/M.*
 - 5.1.2. LAB MEDIA: Jove-Fig2A-raw-data. *Video editor: Highlight the cells showing the flat epithelial-like shape.*
 - 5.1.3. LAB MEDIA: Jove-Fig2B-raw-data. *Video editor: Highlight the round-shaped cells.*
- 5.2. In interphase cells, lipid droplets were distributed throughout the cytoplasm [1]. During metaphase, lipid droplets were preferentially localized outside the bipolar spindle concomitant with cell rounding and chromosome alignment [2].
 - 5.2.1. LAB MEDIA: Figure 3. *Video editor: Show the third panel in the “Merge column” and highlight the green and red colours spread uniformly throughout the cell area.*
 - 5.2.2. LAB MEDIA: Figure 3. *Video editor: Show the fourth panel in the “Merge column”, emphasize the rounded cell at the centre and highlight the green colour surrounding the intense red colour area around the blue colour cell nucleus.*
- 5.3. Validation with higher-resolution imaging showed that lipid droplets were distributed throughout the cytoplasm beneath the cytoskeletal network in interphase cells [1]. During mitosis, particularly at metaphase, lipid droplets were excluded from the spindle region [2] and maintained their spherical morphology [3].
 - 5.3.1. LAB MEDIA: Figure 4. *Video editor: Show the third panel in the “Merge column” and highlight the green dots underlying the red colour fibres spread uniformly throughout the cell area.*
 - 5.3.2. LAB MEDIA: Figure 4. *Video editor: Show the fourth panel in the “Merge column” and emphasize that red colour spindle fibre area is devoid of green colour.*
 - 5.3.3. LAB MEDIA: Figure 4. *Video editor: In the fourth panel in the “Merge column”, emphasize the spherical shape of the green lipid droplets.*

1. HuH7
Pronunciation link: <https://www.merriam-webster.com/dictionary/Huh>
IPA: /'hʌh 'sevən/
Phonetic Spelling: huh·sev·uhn
2. PBS
Pronunciation link: <https://www.howtopronounce.com/pbs>
IPA: /,piː.biː'ɛs/
Phonetic Spelling: pee·bee·ess
3. Asynchronous
Pronunciation link: <https://www.merriam-webster.com/dictionary/asynchronous>
IPA: /ə'sɪŋkrənəs/
Phonetic Spelling: uh·sing·kruh·nuhs
4. Oleic
Pronunciation link: <https://www.merriam-webster.com/dictionary/oleic>
IPA: /oo'liːk/
Phonetic Spelling: oh·lee·ik
5. DMEM
Pronunciation link: No confirmed link found
IPA: /,diː,ɛm,ɛm/
Phonetic Spelling: dee·em·em
6. Millimolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/millimolar>
IPA: /,mɪlə'moʊlər/
Phonetic Spelling: mil·uh·moh·lur
7. Conjugate
Pronunciation link: <https://www.merriam-webster.com/dictionary/conjugate>
IPA: /'kɑːndʒəɡət/, /'kɑːndʒə,ɡɛt/
Phonetic Spelling: kaan·juh·guht
8. Synchronize
Pronunciation link: <https://www.merriam-webster.com/dictionary/synchronize>
IPA: /'sɪŋkrə,naɪz/
Phonetic Spelling: sing·kruh·nyze
9. Micromolar
Pronunciation link: <https://www.howtopronounce.com/micromolar>
IPA: /,maɪkroʊ'moʊlər/
Phonetic Spelling: my·kroh·moh·lur
10. Paraformaldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/paraformaldehyde>
IPA: /,pærəfɔːr'mældə,haɪd/
Phonetic Spelling: pair·uh·for·mal·duh·hyde
11. Permeabilize
Pronunciation link: <https://www.howtopronounce.com/permeabilize>
IPA: /pər'miːə,bɪ,laɪz/
Phonetic Spelling: pur·mee·uh·bih·lyze

12. Triton
Pronunciation link: <https://www.merriam-webster.com/dictionary/Triton>
IPA: /'traɪtɒn/
Phonetic Spelling: try·taahn
13. Alpha Tubulin
Pronunciation link: <https://www.howtopronounce.com/tubulin>
IPA: /'ælfə 'tuːbjəlɪn/
Phonetic Spelling: al·fuh too·byuh·lin
14. Alexa Fluor
Pronunciation link: <https://www.howtopronounce.com/alexa-fluor>
IPA: /ə'leksə 'flɔr/
Phonetic Spelling: uh·lek·suh floor
15. IgG
Pronunciation link: <https://www.howtopronounce.com/igg>
IPA: /,aɪ,dʒi:'dʒiː/
Phonetic Spelling: eye·jee·jee
16. BODIPY 493/503
Pronunciation link: <https://www.howtopronounce.com/bodipy>
IPA: /'boʊdə piː/
Phonetic Spelling: boh·duh·pee
17. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flɔ:'resənt/, /flə'resənt/
Phonetic Spelling: floh·reh·suhnt
18. DAPI
Pronunciation link: <https://www.howtopronounce.com/dapi>
IPA: /'dɑːpi/
Phonetic Spelling: dah·pee
19. Antifade
Pronunciation link: <https://www.howtopronounce.com/antifade>
IPA: /,ænti'feɪd/
Phonetic Spelling: an·tee·fayd
20. Widefield
Pronunciation link: <https://www.howtopronounce.com/widefield>
IPA: /'waɪd fiːld/
Phonetic Spelling: wyde·feeld
21. Interphase
Pronunciation link: <https://www.merriam-webster.com/dictionary/interphase>
IPA: /'ɪntər feɪz/
Phonetic Spelling: in·tur·fayz
22. Mitotic
Pronunciation link: <https://www.merriam-webster.com/dictionary/mitotic>
IPA: /maɪ'tɔːtɪk/
Phonetic Spelling: my·tot·ik

23. Confocal

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

IPA: /kɑːnˈfoʊkəl/

Phonetic Spelling: kaan·foh·kuhl

24. Excitation

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

IPA: /ˌɛksaɪˈteɪʃən/

Phonetic Spelling: ek·sy·tay·shuhn

25. Emission

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

IPA: /ɪˈmɪʃən/

Phonetic Spelling: ih·mish·uhn

26. Cytoplasm

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

IPA: /ˈsaɪtəˌplæzəm/

Phonetic Spelling: sy·tuh·plaz·uhm

27. Metaphase

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

IPA: /ˈmetəˌfeɪz/

Phonetic Spelling: meh·tuh·fayz

28. Bipolar Spindle

Pronunciation link: <https://www.howtopronounce.com/spindle>

IPA: /baɪˈpəʊlər ˈspɪndəl/

Phonetic Spelling: by·poh·lur spin·duhl

29. Concomitant

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

IPA: /kənˈkɑːmətənt/

Phonetic Spelling: kuhn·kaa·muh·tuhnt

30. Cytoskeletal

Pronunciation link: <https://www.howtopronounce.com/cytoskeletal>

IPA: /ˌsaɪtoʊˈskelɪtəl/

Phonetic Spelling: sy·toh·skel·uh·tl

31. Morphology

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

IPA: /mɔːrˈfɑːlədʒi/

Phonetic Spelling: mor·faal·uh·jee

32. Epithelial

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

IPA: /ˌepəˈθiːliəl/

Phonetic Spelling: eh·puh·thee·lee·uhl

33. Chromosome

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

IPA: /ˈkroʊməˌsoʊm/

Phonetic Spelling: kroh·muh·sohm

34. Numerical Aperture

Pronunciation link: <https://www.howtopronounce.com/aperture>

IPA: /nu:ˈmerɪkəl ˈæpərtʃər/

Phonetic Spelling: noo·mair·ih·kuhl ap·ur·chur

35. Organelle

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

IPA: /ˈɔ:rgəˈnel/

Phonetic Spelling: or·guh·nell