Worcester Polytechnic
Institute
100 Institute Road
Worcester, MA 01609-2280
o: 508-831-4961
f: 508-831-5936

Amity L. Manning
Assistant Professor
Biology and Biotechnology
Bioinformatics and Computational Biology

6/24/19

Dear Dr Bajaj,

I thank you and the reviewers for helpful comments on our manuscript. We have addressed each critique fully both through modifications to the text and with the addition of a new figure. The new figure illustrates steps 3.1 through 3.3 in the protocol with a representation of objective and filter cube selection using image acquisition software, use of bright field or phase contrast imaging to identify the plane of focus for the sample that will be imaged, and the consideration of exposure time and pixel binning options. We believe that the suggested revisions have significantly improved the clarity of our manuscript and will make this protocol more useful for those wishing to perform live cell time-lapse imaging. All changes have been indicated by word's 'track changes' feature and point-by-point responses to each critique appear below.

Sincerely,

Amity Manning

Response to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The manuscript has been proofread and all grammatical errors corrected

2. Please provide an email address for each author.
e-mail address for all authors have been added

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”
The short abstract has been revised to reflect the editor’s requested format.
4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Lipofectamine, alisertib, Nikon Ti-E 168 microscope, Nikon NIS194 Elements, epitube, Zyla sCMOS camera, 20x CFI Plan Fluor objective, NIS elements HCA jobs software, etc.
All commercial product names have been removed and are now referenced exclusively in the Table of Materials.
5. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
We have revised the text to ensure it conforms to these guidelines

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
The numbering has been adjusted to follow guidelines provided in the instructions for Authors

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.
Modifications have been made to ensure that the protocol section is written in imperative tense.

8. The Protocol should contain only action items that direct the reader to do something.
Modifications have been made to ensure that the protocol section contains only action items.

9. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
The text has been revised to remove all personal pronouns

10. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
We have ensured that individual protocol steps contain fewer than 3 action steps

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
We have added more detail, as appropriate to describe how individual steps are performed

12. Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).
13. For section C and D, please include knob turns, click-by-click instructions for each software program used. Describe how the user interacts with the software. Click Open, the click Analyze to analyze the image.
To address critique 12 and 13, we have revised the text and added an additional figure (the new figure 1) to include more explicit detail and to emphasize the action to be taken at each step in order to define the image acquisition parameters for live cell time-lapse imaging.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Our protocol and suggested filmable content adhere to these guidelines

Response to Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the manuscript entitled "Live-cell imaging to assess the dynamics of metaphase timing and cell fate following mitotic spindle perturbations", the authors describe methodologies for wide-field fluorescence imaging of mammalian cells as they progress through cell division. The authors describe a procedure for generating cells expressing fluorescently tagged histones and tubulin, as well as plating cells for microscopy. Finally, the authors describe the live cell image acquisition and representative experiments.

The authors nicely emphasize how fixed cell fluorescence or population-based analyses (Western blotting, flow cytometry) poorly capture dynamic processes that happen on a second to minute time scales, and mitosis is one such biological process.

The primary drawback of live cell imaging is that are not particularly hardy specimens on the microscope stage, and great caution must be taken to ensure that any phenotypes observed are due to the applied perturbation and not to issues with cell viability. This manuscript provides a helpful guide to individuals seeking to begin live cell imaging. However, since the image acquisition conditions and settings are somewhat specific to the author's Nikon system, some additional details would be helpful.
As described in response to editorial comments 12 & 13, we have added additional detail and a new figure to illustrate how image acquisition parameters should be selected in

the acquisition software.

Major Concerns:
None.

Minor Concerns:
1. A more detailed description of the environmental chamber should be included. Is it an enclosed microscope stand or a temperature-controlled stage insert? At the very minimum, the system should be shown or described in the video.
We have added a note to protocol step #3.1 to indicate that either an enclosed chamber, or a temperature controlled stage insert would be appropriate for these live cell imaging approaches, provided humidified 5% CO2 and stable temperature control can be obtained. Out lab utilizes an enclosed chamber on our microscope and this will be featured in the video.
2. As underscored by the figures, a 20x dry objectives do not afford high resolution imaging. At a minimum, the authors should list the numerical aperture of their objectives as well as the pixel dimensions of the camera they are using.
We have noted in step #3.2 that the 20x objective used in this protocol has a numerical aperture of 0.5. In step #3.1 we note that at 20x magnification, the high resolution camera used in this protocol captures images with a pixel size of 0.67uM
3. The Addgene catalog numbers should be listed for each construct used.
Addgene numbers have been added for the viral packaging constructs used.

Reviewer #2:
Manuscript Summary:
OK

Major Concerns:
OK

Minor Concerns:
The text is at large clear and easy to follow. However, some improvements are necessary and some information is missing.

General comments.
Although I was not instructed to review the novelty of the method, I think it would be appropriate to cite previous work using similar approaches for time-lapse monitoring of mitosis, e.g., Mackay, Makise and Ullman 2010 J Cell Biol., 191:923 (http://jcb.rupress.org/content/jcb/191/5/923.full.pdf). Otherwise the authors appear as "inventors" of this idea which is largely misleading.
The reviewer makes an excellent point- live cell imaging is a very powerful and widely used approach to assess dynamic cellular behavior! We have added additional citations from the Pellman, Salmon, Khodjakov and Ullman labs to reflect this. While not comprehensive, these citations will provide context for how similar approaches have been employed over the past decade.

In the PDF version is it impossible to follow name of material, company and catalogue number. They should appear on the same page.

The excel sheet has been modified for optimal viewing when converted to a pdf.

Specific comments.
In Figure 3 and corresponding text the authors start using the term NEB (nuclear envelope breakdown). This is misleading since no marker for the NE or nucleocytoplasmic transport cargo have been studied. In preceding figures the authors use prometaphase, based on the appearance of chromatin which fine and fills the same purpose. Thus, NEB should be changed to prometaphase.
While these movies do not specifically employ the use of nuclear envelope markers, the visualization of cytoplasmic-localized tubulin is commonly used to indicate when the nuclear envelope is no longer intact. We have updated the figure legend to indicate that NEB is apparent in the movies as the point in time when tubulin is no longer excluded by the nuclear envelope.

Legend of Figure 3 is confusing concerning the subheading letters. To avoid confusion, letters should appear in the beginning of the sentences (see below).
The figure legend has been modified to address this concern.

Poor resolution in fig 1 A and B, can it be improved?
Image quality was compromised by PDF conversion in the initial submission. High resolution images will be submitted for final review and publication.

The PROTOCOL section can be clearer
We have modified the protocol section and added additional detail to provide the clarity requested by reviewer #2

P2 l 94:
Explain step 1 and 2
The text has been corrected to reflect that "step1" and "step 2" refer to steps 1.1.5 and 1.1.6 respectively

P2, l 104:
How were virus particles concentrated? State centrifugations, titers etc.
A note has been added to protocol step #1.2 to indicate that viral concentration is not required. Viral particles obtained in the manner described will be present in a range of ~1 x10^7 – 1x10^8 Transducing Units /mL. As the viral-producing cells and cells to be infected are both cultured in the same medium, viral concentration to replace medium is not required and filtered viral particles can be used directly to infect the target cell.

P2, l 112:
State end concentration in the wells.
The concentration indicated (8ug/mL) is the end concentration of polybrene (Hexadimethrine bromide) in the wells.

P3, l 153:
Cell adherence, for how long?

Cells lines can vary with respect to the amount of time needed to adhere in preparation for imaging. We have included the following notes to step #2.6 to reflect this and provide the reader guidance when setting up imaging experiments:

> **NOTE: Live cell imaging requires cells to be well-adhered so that they remain associated with the imaging plane during image acquisition. The duration of time needed for cells to adhere following plating can differ from one cell line to the next and should be optimized for the cell line of your choosing.**
>
> **NOTE: Cell seeding concentration should reflect the amount of time your cells will be in culture prior to imaging to ensure that cells do not experience contact-dependent inhibition of proliferation.**

P3, l 158:
How was alisertib dissolved? State volume and final concentration of organic solvent if used.

In response to the Editorial critique we have removed all mention of alisertib (and other commercial products from the protocol, but have included details on preparation of alisertib to the Table of Materials.
In addition, to address the reviewer's comment we have added the following note to indicate that the impact of organic solvents on cellular behavior should be controlled for:

> **NOTE: to account for the potential impact of the organic solvent on cellular behavior, an equal volume of the inhibitor's dilutent should be added to a control well of cells.**

P3, l161:

…in mitotic progression in treated and untreated cultures require…
The text has been revised to clarify the statement

P3, l 165:

Describe microscopy system better. Confocal or not? Objective for imaging? Oil immersion Filters for excitation and emission etc.
Detail has been added to steps #3.1, 3.2, and 3.3 to provide additional detail on the imaging apparatus. Details of the filter cubes used are provided in the Table of Materials

Point 2.2 and 2.3: It is difficult to understand if it is possible to image 5-8 coordinates in 2 channels in 12 wells in 5 minutes. If oil immersion was used, did you have to dispense new oil?
We have clarified that the number of conditions/wells to be imaged will limit the number of coordinates that can be captured per well within the 5 minute timepoint increments in the note added to step 3.4.2 as below:

NOTE: The number of coordinates that can be imaged per well within the 5 minute timepoint increment will be limited by the number of wells to be imaged, as well as the exposure time for each channel.

RESULTS
P5, l 230:
Change "50+ cells" to "at least 50 cells".
This correction has been made.

P7, l310:
Spelling of time lapse
The spelling of time-lapse has been corrected throughout the manuscript.

P7, l330:
Spelling of subsequent
Spelling errors have been identified and corrected

LEGENDS
P6, l 280:
Scale bar is missing in fig 1C.

Reviewer #3:
Manuscript Summary:
This is a very useful contribution for which a video protocol will be welcome by many labs without experience in the technique of live cell imaging. The procedures are well described in the text.

Major Concerns:
1. The authors should emphasize the importance and make explicit the types of controls that are required in these experiments.
We have provided additional notes at steps throughout the protocol to better emphasize where controls should be considered and included.

Minor Concerns:
The following additions are suggested.
1. The authors should indicate how phototoxicity is generally recognized in mitosis experiments, namely by the failure of control cells to complete mitosis.
We have added the following note to step 3.3 to reflect this possibility:

NOTE: Phototoxicity can impair mitotic progression and cell viability. Failure of mitotic cells in control populations to complete normal mitoses may be an indication that exposure times and/or imaging duration needs to be further optimized.

2. Although the authors suggest setting parameters for transmitted light images, they do not show any examples, which viewers may find useful.

We have added a new figure to illustrate the use of bright field or phase contrast imaging to identify and adjust the focus on the cells to be imaged.

3. Although the use of the vital fluorescent DNA dye SiR-Hoechst dye has been criticized as generating DNA damage responses, it and similar vital cytoskeleton dyes remain popular and avoid the necessity for generating cell lines expressing fluorescent proteins, a technique with its own drawbacks. It would be useful to point out this alternative.

This is an excellent point, and we strongly agree that consideration of how a fluorescent tag impacts protein behavior should always be considered. We have added a note in step 1.6 to highlight this concern:

> **NOTE 4: To account for a potential impact of the fluorescent tag on protein behavior and/or for negative impact of the integration site of the viral expression construct on cellular behavior, multiple single cell clones should be derived and tested to monitor that cellular fitness and mitotic progression are not perturbed.**

However, as DNA damage has been linked to mitotic defects and the dynamics of mitotic microtubules have been reported to be altered by live-cell dyes, we caution use of these dyes and favor the use of multiple single cell clones to assess undesirable impact of the fluorescent tag. This too is now reflected in a 'NOTE' on step 1.6

4. It is difficult to determine the quality of the original images from the pdf provided. It would be useful to point out that with the proper accommodation, e.g. an objective warmer, it is possible to carry out high resolution studies with oil-immersion objectives.

While oil immersion objectives would provide greater resolution, it would also limit the number of cells/acquisition field and preclude long-working distance -limiting the imaging platform to coverslip glass. This protocol describes long-term, multifocal time-point image acquisition that is not possible with oil immersion objectives (which would require re-application of oil mid-acquisition).