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Corresponding Author:	Amity Manning WPI Worcester, MA UNITED STATES
Corresponding Author's Institution:	WPI
Corresponding Author E-Mail:	almanning@wpi.edu
Order of Authors:	Dayna L Mercadante
	Elizabeth A Crowley
	Amity Manning
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TITLE:

Live Cell Imaging to Assess the Dynamics of Metaphase Timing and Cell Fate Following Mitotic Spindle Perturbations

AUTHORS & AFFILIATIONS:

Dayna L Mercadante[1*], Elizabeth A Crowley[1*], Amity L Manning[1]

[1]Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, MA, USA

[*]These authors contributed equally

Corresponding author:
Amity L Manning (almanning@wpi.edu)

Email Addresses of Co-Authors:
Dayna L Mercadante (dmercadante@wpi.edu)
Elizabeth A Crowley (eacrowley@wpi.edu)

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SUMMARY:
Here we present a protocol to assess the dynamics of spindle formation and mitotic progression. Our application of time-lapse imaging enables the user to identify cells at various stages of mitosis, track and identify mitotic defects, and analyze spindle dynamics and mitotic cell fate upon exposure to anti-mitotic drugs.

LONG ABSTRACT:
Live cell time-lapse imaging is an important tool in cell biology that provides insight into cellular processes that might otherwise be overlooked, misunderstood, or misinterpreted by the fixed-cell analysis. While the fixed cell imaging and analysis is robust and sufficient to observe cellular steady-state, it can be limited in defining a temporal order of events at the cellular level and is ill-equipped to assess the transient nature of dynamic processes including mitotic progression. In contrast, live cell imaging is an eloquent tool that can be used to observe cellular processes at the single-cell level over time and has the capacity to capture the dynamics of processes that would otherwise be poorly represented in fixed cell imaging. Here we describe an approach to generate cells carrying fluorescently labeled markers of chromatin and microtubules and their use in live cell imaging approaches to monitor metaphase chromosome alignment and mitotic exit. We describe imaging-based techniques to assess the dynamics of spindle formation and mitotic progression, including the identification of cells at various stages in mitosis, identification, and tracking of mitotic defects, and analysis of spindle dynamics and mitotic cell fate following the treatment with mitotic inhibitors.

INTRODUCTION:

Image-based analysis of fixed cells is commonly used to assess the cell population level changes in response to various perturbations. When combined with cell synchronization, followed by the collection and imaging of serial time points, such approaches can be used to suggest a cellular sequence of events. Nevertheless, fixed cell imaging is limited in that temporal relationships are implied for a population and not demonstrated at the level of individual cells. In this way, while fixed cell imaging and analysis is sufficient to observe robust phenotypes and steady-state changes, the ability to detect transient changes over time and changes that impact only a subpopulation of the cells is imperfect. In contrast, live cell imaging is an eloquent tool that can be used to observe cellular and subcellular processes within a single cell, or cellular population, over time and without the aid of synchronization approaches that may themselves impact cellular behavior[1-6].

The formation of a bipolar mitotic spindle is essential for the proper chromosome segregation during cell division, resulting in two genetically identical daughter cells. Defects in mitotic spindle structure that corrupt mitotic progression and compromise the fidelity of chromosome segregation can result in catastrophic cell divisions and reduced cell viability. For this reason, mitotic poisons that alter spindle formation are promising therapeutics to limit the rapid proliferation of cancer cells[7-9]. Nevertheless, fixed cell analysis of spindle structure following the addition of mitotic poisons is limited in its ability to assess the dynamic process of spindle formation and may not indicate whether observed changes in spindle structure are permanent or are instead transient and may be overcome to permit successful cell division.

In this protocol, we describe an approach to assess the dynamics of mitosis following spindle perturbations by live cell imaging. Using the hTERT immortalized RPE-1 cell line engineered to express an RFP-tagged Histone 2B to visualize chromatin, together with an EGFP-tagged α-tubulin to visualize microtubules, the timing of metaphase chromosome alignment, anaphase onset, and ultimately mitotic cell fate are assessed using visual cues of chromosome movement, compaction, and nuclear morphology.

PROTOCOL:

1. Generation of hTERT-RPE-1 cells stably expressing RFP-Histone 2B (RFP-H2B) and α-tubulin-EGFP (tub-EGFP)

NOTE: All steps follow aseptic techniques and take place in a biosafety level II+ (BSL2+) safety cabinet.

1.1. Generate retrovirus carrying the genes of interest (α-tubulin-EGFP and RFP-H2B) by the transfection of 293T cells with the appropriate lentiviral plasmids according to the manufacturer's instructions of the lipid-based transfection delivery system.

1.1.1. Day 1: Use a disposable glass Pasteur pipette to aspirate the cell culture medium from a plate of sub-confluent 293T cells and wash off the residual medium with phosphate buffered saline (PBS) by adding 5 mL of PBS. Swirl to distribute PBS over the plate bottom, then aspirate the PBS with a sterile disposable glass Pasteur pipette.

1.1.2. Add 2 mL of 0.05% trypsin that has been pre-warmed to 37 °C and return the plate to a humidified incubator at 37 °C with 5% CO_2 for 2 to 5 min to allow adherent cells to be released from the cell culture dish surface.

1.1.3. Add 8 mL of fresh Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin to the plate containing trypsin.

1.1.4. Resuspend the cells by gently pipetting and transfer the suspension to a sterile 15 mL conical tube. Place the conical tube in a balanced centrifuge and spin at 161 *x g* for 5 min at room temperature to gently pellet the cells

1.1.5. Aspirate the medium/trypsin solution from pelleted cells and resuspend cells in 10 mL of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Use a hemocytometer to count the 293T cell suspension and plate 2 x 10^6 293T cells per well of a 6 well plate.

1.1.6. Culture cells in a total volume of 2 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained in a humidified incubator at 37 °C with 5% CO_2.

1.1.7. Day 2: Pipette 7 µL of lipid-based transfection delivery reagent and 100 µL of reduced serum medium into a 1.5 mL microcentrifuge tube and allow it to incubate at room temperature for 5 min (tube #1).

1.1.8. In a separate 1.5 mL microcentrifuge tube, pipette 1 µg of RFP-H2B expression vector (or 1 µg of α-tubulin-EGFP expression vector), together with 5 µL enhancer reagent (as required per manufacturer's guidelines), 0.5 µg pMD2.G, and 1 µg psPAX2 and 100 µL of reduced serum medium (tube #2).

1.1.9. Combine the contents of step 1.1.7 and step 1.1.8 by carefully pipetting tube 2 into tube #1 and incubate for 20 min at room temperature.

NOTE: The reaction can be scaled as needed for transfection of cells in additional wells.

1.1.10. Pipette the transfection reaction dropwise to the desired well containing 293T cells in 2 mL of medium and return the dish to the humidified incubator at 37 °C with 5% CO_2.

NOTE: To generate cells expressing both RFP-H2B and α-tubulin-EGFP, generate separate virus for each expression construct (steps 1.1.7- 1.1.9).

1.1.11. Day 3: Aspirate and replace the medium with 2 mL of fresh DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Return the dish to the humidified incubator at 37 °C with 5% CO_2.

1.2. Day 4: Collect the medium containing expressed virus particles, being cautious not to disrupt or remove 293T cells. Filter the medium containing virus particles by passing it through a 0.45 µM filter attached to a 5 mL syringe. Aliquot virus for short term storage at 4 °C, or long term storage at -80 °C.

NOTE: Viral particles obtained in this manner will be present in a range of ~1 x 10^7 to 1 x 10^8 Transducing Units/mL. As the viral-producing cells and cells to be infected are both cultured in the same medium, viral concentration to replace the medium is not required and filtered viral particles can be used directly to infect cells.

1.3. Seed 2 x 10^5 hTERT-RPE-1 cells per well of a 6-well dish in preparation for the viral infection. Culture cells in 2 mL of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and maintain in a humidified incubator at 37 °C with 5% CO_2.

1.4. Day 5: Add hexadimethrine bromide (e.g., polybrene) to the hTERT-RPE-1 cells to a final concentration of 8 µg/mL. Infect cells with a mixture of 500 µL of virus diluted in 500 µL of cell culture medium.

NOTE: Hexadimethrine bromide stock solution is prepared in sterile distilled water, then filtered through a 0.45 µm filter.

1.5. Day 6: Using a disposable glass Pasteur pipette, aspirate the medium from wells containing the virus infected cells. Replace the cell culture medium with 2 mL of DMEM containing 10% FBS, 1% penicillin/streptomycin, and appropriate concentrations of antibiotic to select for the plasmid integration and expression.

NOTE: The α-tubulin-EGFP expression plasmid used in the described experiments carries a puromycin-resistance gene and the RFP-H2B expression plasmid carries a blasticidin resistance gene. Therefore, 10 µg/mL of puromycin and 2 µg/mL of blasticidin are used for the selection of α-tubulin-EGFP, RFP-H2B expressing hTERT RPE-1 cells. Concentrations of antibiotic used for the selection may differ for various cell lines used.

1.6. Maintain cells under antibiotic selection for 5-7 days, replacing the medium every 3 days with fresh medium containing appropriate selection reagents.

NOTE: Cells should be maintained at sub-confluence during the selection and should be expanded as needed, as described in steps 1.1.1 to 1.1.4.

1.7 Use immunofluorescence imaging to confirm the expression of tagged constructs.

NOTE: If desired, single cell clones can be derived to obtain uniform expression levels within the cell population. Once stable clones are confirmed, cells can be maintained under the standard culture conditions in the absence of antibiotic selection.

2. Preparation of cells for live cell imaging following mitotic spindle perturbations

NOTE: Use aseptic techniques and perform the steps in a BSL2 safety cabinet.

2.1 Using a sterile disposable glass Pasteur pipette, aspirate the medium from the culture plate containing the cell line that carries the expression construct(s) (from step 1.7). Briefly wash the cells with 10 mL of sterile PBS. Swirl to distribute PBS over the plate bottom, then aspirate PBS with a sterile disposable glass Pasteur pipette.

2.2 Add 2 mL of 0.05% trypsin to the 10 cm plate. Incubate the plate at 37 °C for 2-5 min or until cells have detached from the plate surface.

2.3 Add 8 mL of fresh medium to the plate containing trypsin. Resuspend the cells by gently pipetting and transfer the suspension to a sterile 15 mL conical tube. Place the conical tube in a balanced centrifuge and spin at 161 *x g* for 5 min at room temperature to gently pellet the cells.

2.4 Carefully aspirate the supernatant and resuspend in 10 mL of PBS by pipetting gently with a 10 mL serological pipette. Place the conical tube in a balanced centrifuge and spin at 161 *x g* for 5 min at room temperature to gently pellet the cells.

2.5 Carefully aspirate the supernatant and resuspend in 10 mL of the fresh medium by pipetting gently with a 10 mL serological pipette.

2.6 Count cells, then calculate the cell number using a hemacytometer and dilute to a concentration of 1-2 x 10^5 cells/mL in the cell culture medium. Seed 500 µL of the cell suspension to each well of a sterile 12-well imaging bottom plate. Place the plate in the cell culture incubator and allow cells to adhere to the plate surface.

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 the 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 under study.

2.7 Up to 30 min prior to initiating time-lapse imaging, add a relevant concentration of a mitotic drug to one or more of the wells seeded with cells. To account for the potential impact of the organic solvent on cellular behavior, add an equal volume of the inhibitor's diluent to cells as controls. For example, ensure that the addition of 100 nM of the specific inhibitor of the mitotic kinase Aurora A (e.g., alisertib) is paralleled with an equal volume of DMSO, the diluent for this drug, in a control well of cells.

NOTE: Comparative analysis of dynamic changes in the mitotic progression require wells of cells to be prepared in the absence of perturbations to enable imaging of normal mitoses in parallel to experimental conditions.

3. Microscope set up for time-lapse imaging of RFP-H2B, α-tubulin-GFP expressing cells (Figure 1, Figure 2)

3.1 Place the cell culture plate containing RFP-H2B, α-tubulin-GFP expressing hTERT RPE-1 cells to be imaged into an appropriate stage insert on an inverted epifluorescence microscope that is equipped with a high resolution camera (pixel size of 0.67 μm at 20x), an environmental chamber preheated to 37 °C, and the delivery system for humidified 5% CO_2.

NOTE: Either enclosed environmental chambers or temperature controlled stage inserts may be appropriate provided humidified 5% CO_2 can be provided and stable temperature control obtained.

3.2 Use a 20x air objective with a numerical aperture of 0.5 and equipped for the high contrast fluorescence and phase contrast or brightfield imaging. View cells with the phase contrast or brightfield and adjust the course and fine focus on the microscope to bring cells into focus.

3.3 Identify and set the optimal exposure times for brightfield, GFP, and RFP image acquisition by selecting the respective filter cube with appropriate excitation and emission for the fluorophores that will be imaged. Alternatively, click the auto exposure button on inputting a predetermined exposure time. If the signal is not sufficiently intense, select pixel binning to enable shorter exposure times by clicking on the **binning** tab and selecting **2 x 2 pixel binning** from the drop down menu.

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.

3.4 Use an acquisition and analysis software that enables multi-coordinate, multi-well imaging to be acquired concurrently and define the parameters for the image acquisition.

3.4.1. Select and calibrate the microscope stage to the multi-well dish, according to the manufacturer's instructions. Use the image acquisition software to highlight or otherwise select the wells that will be imaged by clicking on the relevant wells in the diagram of the plate format that is being used.

3.4.2. Under the **GeneratedPoints** control panel (**Figure 2**), define the coordinates within each well that will be imaged by clicking on the **Point Placement** tab and selecting **predefined** or **random coordinate placement** from the drop down menu. Select the **Working Area** tab and select **restricted** from the drop down menu to restrict the coordinate selection area to exclude

the boundaries of the well. Click the **Count** and **Distribution** tabs to select the number and distribution of points to be captured per well, respectively.

NOTE: The number of coordinates that can be imaged per well within the 5 min timepoint increment will be limited by the number of wells to be imaged, as well as the exposure time for each channel. Typically, 5-8 coordinates per well are sufficient to observe at least 50 cells in each condition progress through mitosis within 4 h.

3.4.3. Select and input the time interval and duration to collect images by clicking on and inputting the values in the **TimeSequence** control panel.

NOTE: Time-lapse imaging, acquiring images in each channel every 1 to 5 min is appropriate to monitor the dynamics of mitotic progression and the duration of imaging should reflect the desired endpoint and proliferation rate of the cell line. 4 h is sufficient to see many cells progress through mitosis, 16 h is sufficient to see most RPE-1 cells in asynchronous population progress through mitosis.

4. Time-lapse image analysis to determine metaphase timing and mitotic cell fate following mitotic spindle perturbations

NOTE: Perform the image analysis using an image acquisition software (**Table of Materials**), ImageJ, or comparable image analysis software.

4.1 Visualize RFP-H2B labeled chromatin by selecting the images captured with the RFP filter cube in place. Identify a cell entering mitosis as indicated by initial chromatin compaction (**Figure 2C**, blue arrowhead) and nuclear envelope break down (when α-tubulin-EGFP is no longer excluded by the nuclear boundary).

4.2 Determine the mitotic timing of metaphase alignment and anaphase onset in individual cells: Track the cell through consecutive timepoints in the acquired movie to determine the number of timepoints/minutes from mitotic entry until RFP-H2B-labelled chromatin completes alignment at the cell equator during metaphase (**Figure 2C**, yellow arrowhead).

4.3 To monitor mitotic timing, mitotic fidelity, and cell fate, continue to track the cell through consecutive timepoints to identify the time coordinate at which anaphase chromosome segregation is apparent (**Figure 2C**, white arrowhead) and/or where chromatin decompaction and nuclear envelope reformation (as indicated by tubulin exclusion from the nucleus) has occurred.

NOTE: Perturbations in spindle assembly or mitotic progression can be assessed as a function of the time required to obtain a bipolar mitotic spindle and achieve the chromosome alignment, or to complete anaphase chromosome segregation.

4.4 Visualize RFP-H2B to identify cells in each population that exhibit mitotic defects including lagging chromosomes and chromatin bridges during anaphase chromosome segregation.

NOTE: Compromised mitotic fidelity may also result in multinucleated or micronucleated daughter cells and can be visualized in cells post mitotic exit, as in **Figure 3**.

REPRESENTATIVE RESULTS:

Assessment of mitotic progression in the presence of spindle perturbations

The regulation of spindle pole focusing is an essential step in proper bipolar spindle formation. Disruption in this process through protein depletions, drug inhibition, or alterations in centrosome number corrupt spindle structure and delay or halt mitotic progression[10-13]. Nevertheless, some perturbations only transiently delay spindle formation with cells ultimately proceeding through mitosis to complete anaphase chromosome segregation[14]. In the absence of cell synchronization approaches, which may themselves indirectly impact mitotic processes[1,2], cells progress through mitosis asynchronously (**Figure 2C**). Using RFP-H2B to identify chromatin, mitotic cells with compact chromatin can be staged as being in prometaphase (those prior to complete metaphase alignment: **Figure 2C**, blue arrowheads), metaphase (complete chromosome alignment: **Figure 2C**, yellow arrowheads) and anaphase (chromosomes being segregated towards spindle poles: **Figure 2C**, white arrowheads). The capture of 5-8 coordinates over 4 h is sufficient to follow the progression of at least 50 cells through mitosis in a given condition. To investigate the dynamics of spindle assembly following alterations in centrosome number and/or inhibition of Aurora A kinase, an important regulator of spindle pole focusing[14-17], we used the live cell time-lapse imaging of human cells with 2 centrosomes, or those containing supernumerary centrosomes. Cells were cultured in the presence or absence of the Aurora A kinase inhibitor prior to the start of imaging. Cells with 2 centrosomes experience the disruption in mitotic progression when treated with aurora A kinase inhibitor, but are ultimately able to achieve metaphase chromosome alignment (**Figure 2C**, yellow arrowhead). In contrast, cells with >2 centrosomes are exquisitely sensitive to Aurora A inhibition and exhibit an increase in prometaphase cells and an absence of metaphase cells, indicating a delay in spindle assembly and mitotic progression.

Figure 3 demonstrates that such time-lapse imaging approaches are sufficient to monitor both spindle assembly and mitotic fidelity. By visualizing α-tubulin-EGFP, it was observed that cells that experience spindle disruption (shown here due to centrosome overduplication) undergo dynamic changes as spindle pole focusing is achieved and a bipolar mitotic spindle is formed in preparation for cell division. Concurrent with spindle assembly, chromosome movement can be visualized with RFP-H2B to assess the chromosome alignment and segregation fidelity. Mitotic cells that experience transient spindle multipolarity are susceptible to the attachment errors that result in lagging chromosomes during anaphase and form micronuclei in the subsequent G1 phase of the cell cycle. Such defects are apparent with these live cell imaging approaches.

Changes in the dynamic progression of mitosis alter mitotic timing and impact mitotic cell fate

Following the nuclear envelope breakdown, spindle formation and chromosome movement can

be tracked through the stages of mitosis to assess mitotic progression, duration, and the fate of cells that progress into anaphase. Centrosome amplification, a feature common in many types of cancer, is characterized by the presence of extra centrosomes and multipolar spindle formation[18-20]. As multipolar divisions result in highly aneuploid and likely unviable daughter cells, cancer cells actively cluster extra centrosomes to form a bipolar spindle and undergo a bipolar division[5,20-24]. Using live cell imaging approaches, our representative results show that cells with a normal centrosome content are able to proceed from nuclear envelope breakdown through metaphase alignment and anaphase onset to achieve a bipolar division in under 30 minutes (**Figure 4A,D,E**). In the presence of extra centrosomes, nearly 50% of cells are able to overcome a transient multipolar mitotic spindle and to form a bipolar spindle and complete cell division (**Figure 4B,D**). The remaining cells are unable to achieve a bipolar spindle and as a result exit mitosis through a multipolar division (**Figure 4C,D**). Regardless of whether spindle bipolarity is achieved, cells with extra centrosomes exhibit a significantly increased duration of mitosis compared to cells with 2 centrosomes, indicating that the dynamics of mitotic progression may be altered even when changes in mitotic outcome are not apparent (**Figure 4E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Selection of microscope and camera parameters. (**A**) Representation of the window to select the desired objective and appropriate filter cube using an image acquisition software. Shown is the selection of the 20x magnification objective and the brightfield filter cube. (**B**) Cells in the sample plate are found and brought into focus using brightfield or phase contrast microscopy. (**C**) Representation of the window to set exposure parameters for each image capture. Pixel binning can be used, if needed, to achieve reduce exposure time per capture and minimize photo-damage to cells. Scale bar is 10 μm.

Figure 2: Image capture and analysis of time-lapse microscopy. (**A** and **B**) Representation of the window indicating how image acquisition parameters are set using NIS Elements HCA jobs image acquisition software. This software allows multi-coordinate, multi-well imaging over time. Each job can be modified to specify wells and coordinates to be captured. (**C**) Single time frames from four conditions of one experiment. RFP-H2B is used to track chromatin. Chromatin compaction and positioning are used to identify different stages of mitosis: Prometaphase (compaction in the absence of chromosome alignment, blue arrowhead), Metaphase (complete chromosome alignment at the cell equator, yellow arrowhead), and Anaphase (following initiation of synchronous chromosome segregation, white arrowhead). Scale bar is 10 μm.

Figure 3: Time-lapse imaging visualizes defects in mitotic fidelity. Cells containing extra centrosomes form multipolar spindles and often cluster them into a bipolar spindle prior to completing cell division. Here, a cell enters mitosis with seven distinct spindle poles (white asterisks) which, over time, are clustered into two main spindle poles. At this point, chromosomes are able to align along the spindle equator, and the cell proceeds to anaphase. The transient multipolarity has permitted a mal attachment of a single chromosome. This unresolved error results in a lagging chromosome during anaphase which becomes incorporated into a micronucleus in one daughter cell (labeled with an arrow). Chromatin is visualized with RFP-

Histone 2B and microtubules are visualized with α-tubulin-EGFP. Time stamps in each panel indicate minutes with respect to apparent nuclear envelope breakdown as judged by detection of tubulin within the nuclear boundary. Scale bar is 5 µm.

Figure 4: Changes in the dynamic progression of mitosis alter mitotic timing and impact mitotic cell fate. RFP-H2B, shown in red, is used to track chromatin movement and α-tubulin-GFP, shown in green, is used to monitor centrosome/spindle pole number and organization. Loss of GFP-tubulin exclusion from the nucleus, concurrent with early chromatin compaction is an indication that the nuclear envelope has broken down (NEB) in preparation for mitotic cell division. Nuclear exclusion of GFP-tubulin is apparent upon mitotic exit and reformation of the nuclear envelope. (**A**) A cell with two centrosomes forms a bipolar spindle and progresses through anaphase to form two daughter cells. (**B and C**) Cells with extra centrosomes enter mitosis to form a multipolar spindle. (**B**) Some of these cells with extra centrosomes delay in mitosis without achieving a bipolar spindle and progress to complete multipolar anaphase. (**C**) Other cells with extra centrosomes exhibit a transient delay in mitotic progression while they cluster extra centrosomes to enable bipolar anaphase**.** (**D**) Cells with extra centrosomes are able to undergo a bipolar division approximately 50% of the time, while the remaining cells with extra centrosomes undergo a multipolar division. (**E**) Cells with extra centrosomes have increased mitotic timing regardless of the eventual mitotic fate (bipolar or multipolar anaphase). Scale bar is 5 µm.

DISCUSSION:

The temporal resolution provided by the time-lapse imaging allows for the visualization and assessment of sequential cellular events within single cells. Approaches that make use of cellular synchronization followed by the collection and fixation of cells at sequential time points are limited in that comparisons are ultimately made between populations of cells. In contexts where the cellular response to perturbations may be non-uniform, or where the process being visualized is dynamic, live cell time-lapse imaging is better equipped to follow and analyze both the dynamics of single cells, as well as the heterogeneity within a cellular population. In this way, time-lapse imaging is particularly useful in monitoring cell progression through the dynamic stages of mitosis and assessing how perturbations to this progression ultimately impact the fidelity of mitotic cell division and subsequent cell fate.

While there are a number of advantages to live cell imaging, significant challenges are associated that must be considered and mitigated when possible. One challenge is in maintaining appropriate environmental conditions to ensure cell viability and proliferation during imaging. To accomplish this, the imaging set-up must include an environmental chamber that regulates both temperature and CO_2. Alternatively, cells may be imaged for a short-term with only temperature regulation if done so in a closed chamber. In both cases, use of an antivibration table and/or hardware-based approaches to mitigate axial focus fluctuations (e.g., Nikon's Perfect Focus) should be employed to maintain plate focus throughout the duration of the experiment. A second significant concern with live cell imaging is the potential for photodamage and photobleaching. Photobleaching is a concern where the fluorophore being imaged gradually decreases in fluorescence intensity as imaging progresses. However, the exposure to high intensity excitation light that results in photobleaching is also toxic to cells and care must be made to minimize cell

exposure by decreasing exposure times, image acquisition intervals, and the total duration of the imaging sequence[25]. Consequences of not optimizing imaging conditions to minimize phototoxicity can include the generation of DNA damage and other cellular changes that can in turn compromise interpretation and understanding of the experiment. Should cell viability become a concern with long-term imaging, effort should be made to utilize pixel binning (to enable shorter exposures) and increase the duration between subsequent image captures. An additional concern is that the fluorescent tag may alter the behavior of the protein to which it is fused, or that the integration of the viral expression construct into the genome may itself impact cellular behavior. To account for the possibility that the addition of a fluorescent tag may impact protein function, an assessment of protein function following the addition of an N or C terminal fluorescent tag and comparison with non-tagged protein function is necessary. To determine if adverse effects on cell behavior arise due to the perturbation of the genomic locus in which the viral construct has been integrated, multiple single cell clones should be derived, compared to cells that lack the tagged protein, and tested to monitor that cellular fitness and mitotic progression are not perturbed. Alternatively, off target effects of random integration of the viral expression construct can be mitigated through targeted integration of the fusion protein into the endogenous locus, or other known regions of the genome, using CRISPR-based approaches.

Approaches to identify and characterize major regulators of mitotic progression have relied heavily on fixed cell imaging. Mitotic regulators identified in this way have subsequently been exploited in therapeutics targeting rapidly proliferating cancer cells[7-9]. However, antimitotic drugs do not always perform uniformly in different cancers and in many cases insight into the mitotic phenotypes that precede either successful or unsuccessful chemotherapeutic approaches remain unclear. Live cell time-lapse imaging to track mitotic cells in the presence and absence of spindle-perturbing drugs has the potential to provide the insight necessary to identify those modulators most likely to result in catastrophic mitoses and compromised viability of cancer cells with minimal impact on normal cells.

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The authors have nothing to disclose.

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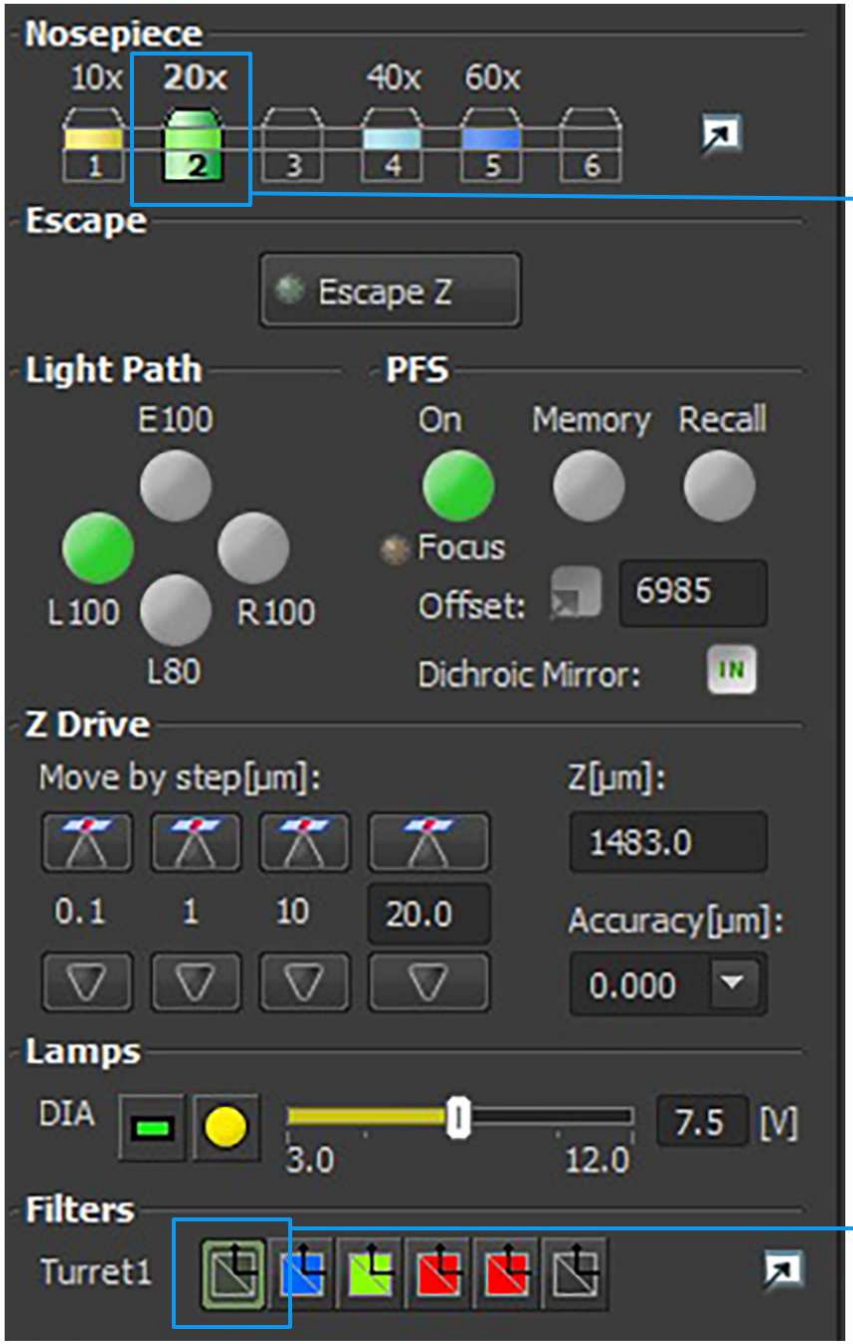
531

A



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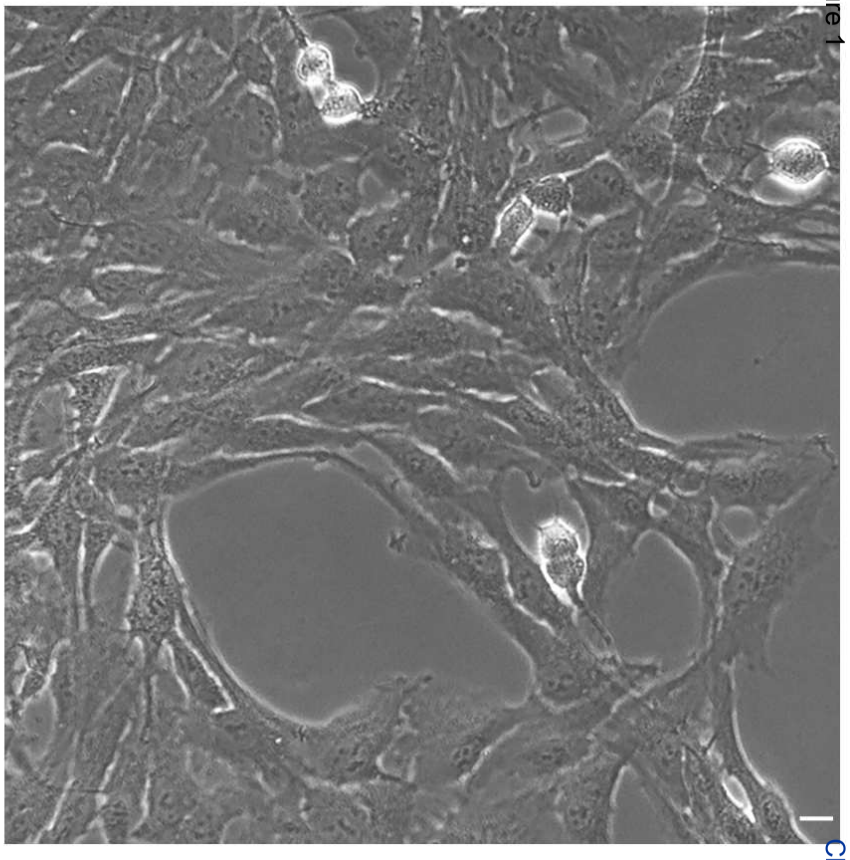

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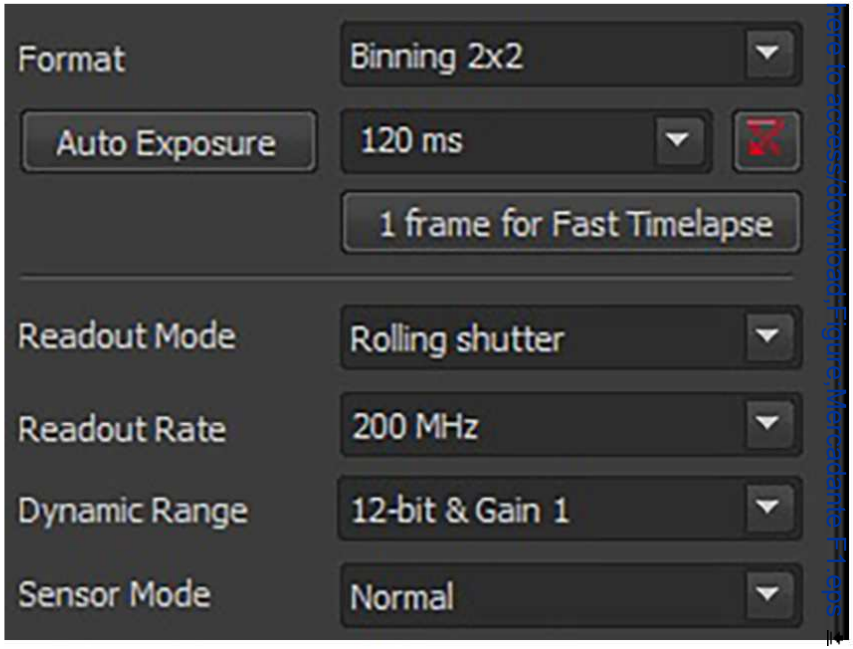

Figure 2

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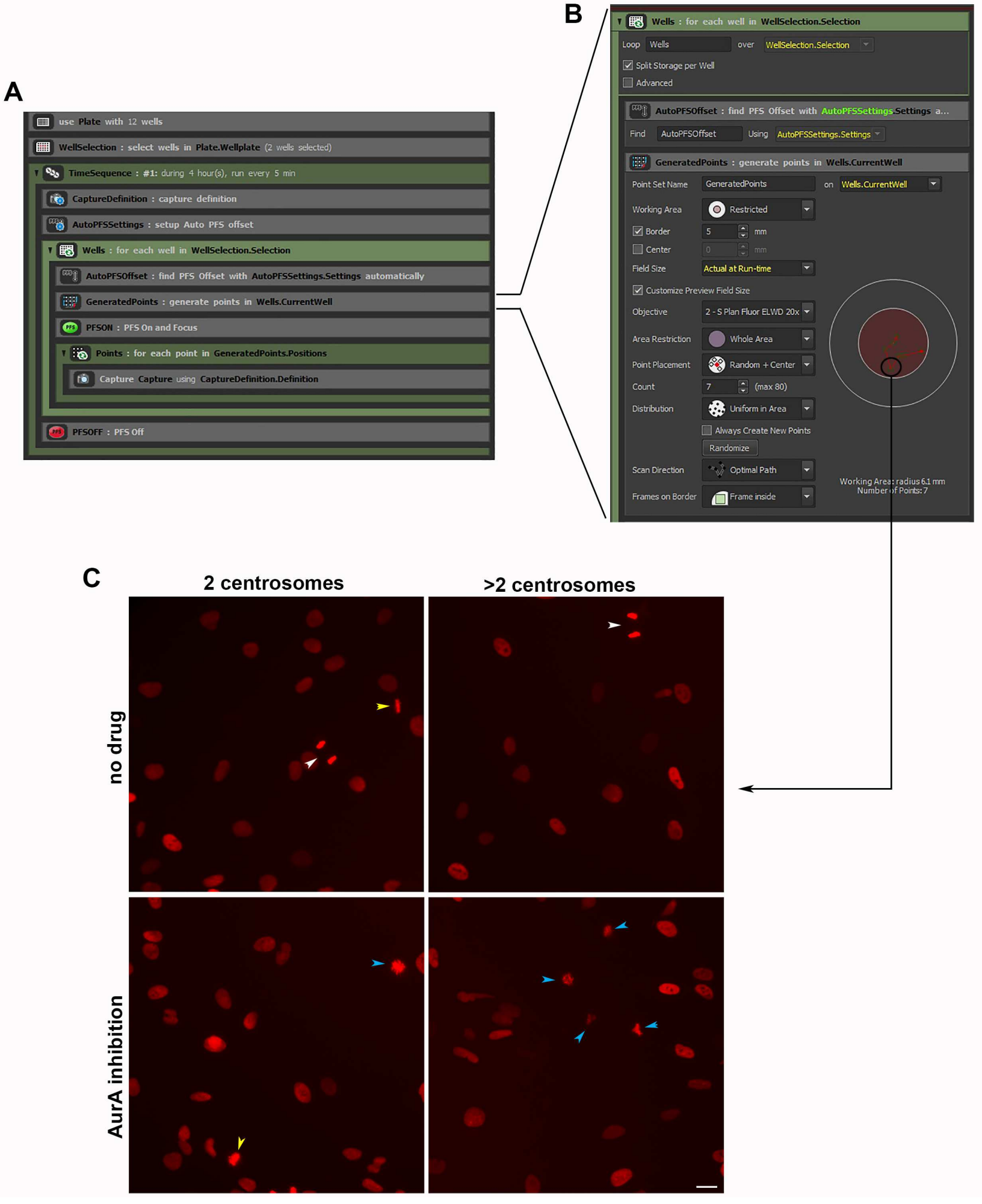

Figure 3

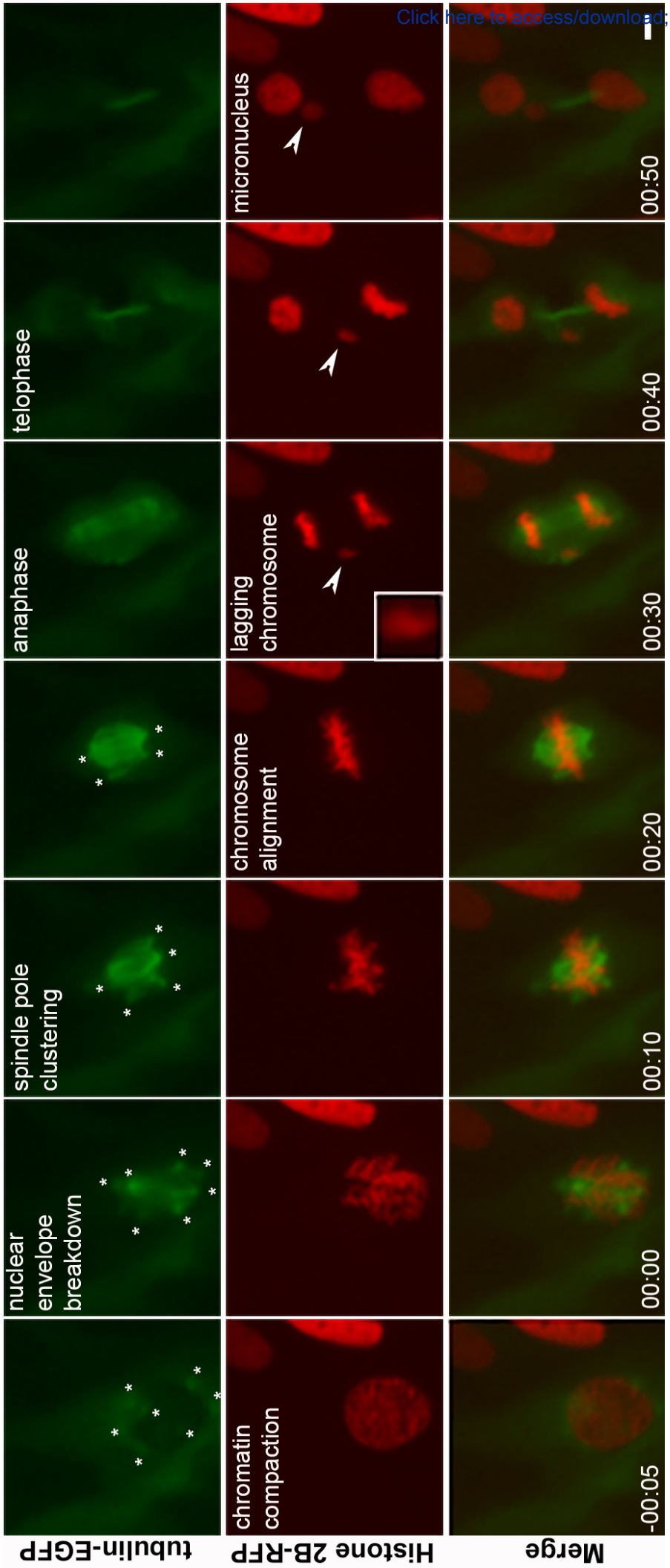

Figure 4

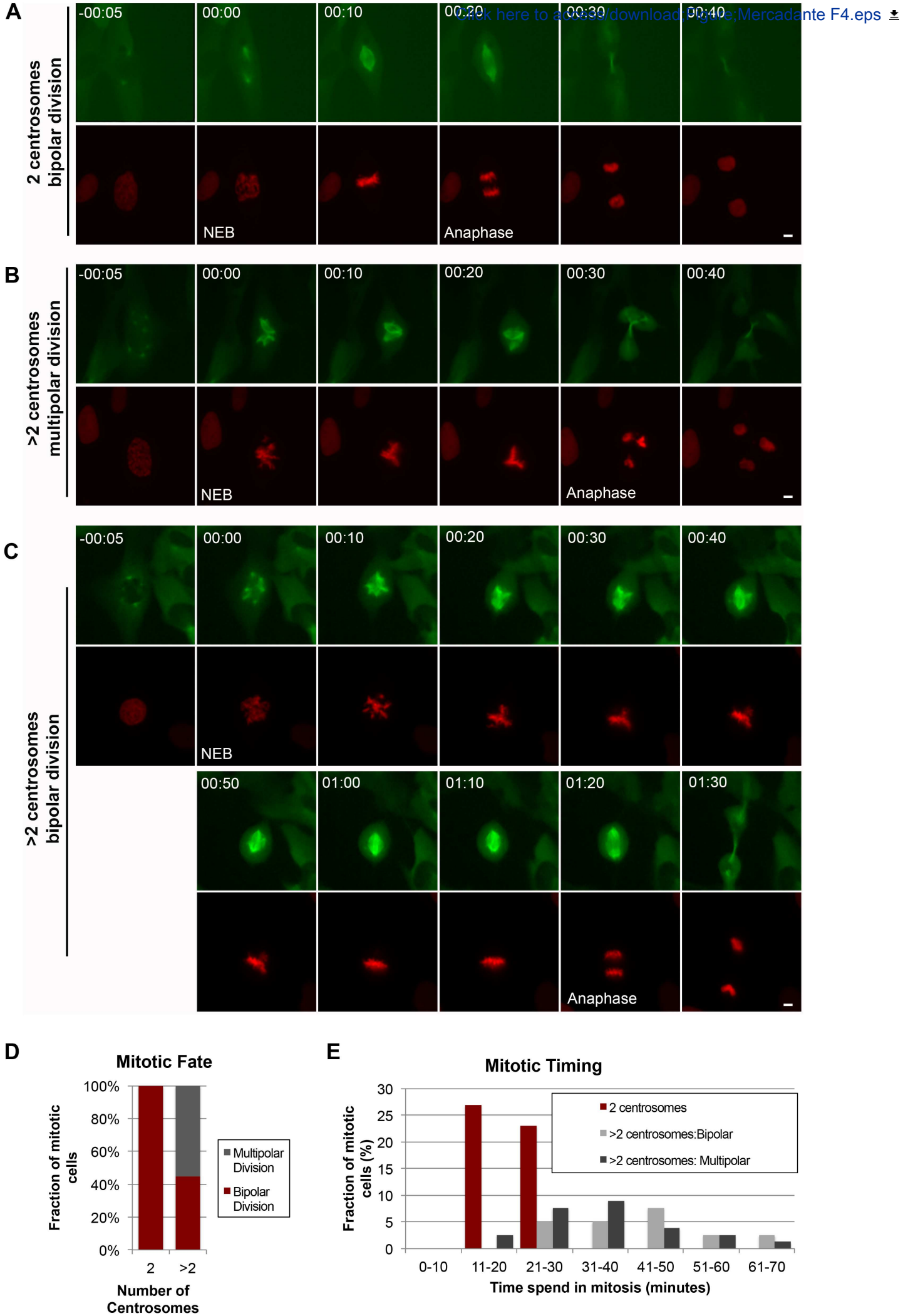

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.05% Trypsin	Gibo-Life sciences	25-510	A serine protease used to release adherent cells from culture dishes
15ml centrifuge tubes	Olympus Plastics	28-101	
20x CFI Plan Fluor objective	Nikon		For use in Live-cell imaging to visualize both bright field and fluorescence
293T Cells	ATCC	CRL-3216	For use in retroviral transfection; used in step 1.1
a-tubulin-EGFP	Addgene	various numbers	Expression vector for alpha tubulin fused to a green fluorescent protein tag; for use in the visualization of tubulin in live-cell imaging: commercially available through addgene and other vendors
Alisertib	Selleckchem	S1133	Small molecule inhibitor of the mitotic kinase Aurora A. Stock concentration is prepared at 10mM in DMSO, and used at a final concentration of 100nM.
Blasticidin	Invitrogen	A11139-03	Antibiotic selection agent; used to select for a-tub-EGFP expressing cells
C02	Airgas		For use in cell culture and live cell imaging
Chroma ET-DS Red (TRITC/Cy3)	Chroma	49005	Single band filter set; excitation wavelength 545nm with 25nm bandwidth and emission at 605nm wavelength with 70nm bandwidth; for visualization of H2B-RFP
Chroma ET-EGFP (FITC/Cy2)	Chroma	49002	Single band filter set; excitation wavelength 470nm with 40nm bandwidth and emission at 525nm wavelength with 50nm bandwidth; for visualization of GFP-tubulin
disposable glass Pastuer pipets, sterilized	Fisher Scientific	13-678-6A	For use in aspirating cells
Dulbecco's Modified Eagle Medium (DMEM)	Gibo-Life sciences	11965-084	Cell culture medium for growth of RPE-1 and 293T cells
Fetal Bovine Serum (FBS)	Gibo-Life sciences	10438-026	Cell culture medium supplement
Lipofectamine 3000 and p3000	Invitrogen	L3000-015	Lipid based transfection reagent for transfection of plasmids; used in 1.1.4
Multi well Tissue Culture dishes	Corning	various	for use in cell culture, transfection/infection, and live cell imaging
Nikon Ti-E microscope	Nikon		Inverted epifluorescence microscope for use in live-cell imaging
NIS Elements HC	Nikon	Version 4.51	Image acquisition and analysis software; used in sections 3 & 4
OPTI-MEM	Gibo-Life sciences	31985-070	Reduced serum medium for cell transfection; used in step 1.1.3
Penicillin/Streptomycin	Gibo-Life sciences	15140-122	antibiotic used in cell culture medium
phosphate bufferred saline (PBS)	Caisson labs	PBP06-10X1LT	sterile saline solution for use with cell culture
pMD2.G	Addgene	12259	Lentiviral VSV-G envelope expression construct; used in step 1.1.4
Polybrene	Sigma-Aldrich	H9268	Cationic polymer used to enhane viral infection efficiency; used in step 1.1.10
psPAX	Addgene	12260	2nd generation lentiviral packaging plasmid; used in step 1.1.4
Puromycin	Invitrogen	ant-pr-1	Antibiotic selection agent; used to select for RFP-H2B expressing cells
RFP- Histone 2B (H2B)	Addgene	various numbers	Expression vector for red fluorescent protein-tagged histone 2B; for use in the visualization of chromatin in live-cell imaging: commercially available through Addgene and other vendors
RNAi Max	Invitrogen	13778-150	Lipid based transfection reagent for transfection of siRNA constructs
RPE-1 cells	ATCC	CRL-4000	Human retinal pigment epithelial cell line
Tissue culture dish 100x20mm	Corning	353003	for use in culturing adherent cells
Zyla sCMOS camera	Nikon		Camera attached to the micrscope, used for capturing images of cells

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Author(s):	Dayna L Mercadante, Elizabeth A Crowley, and Amity L Manning

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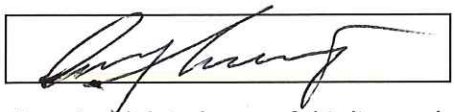
Name:	Amity Manning
Department:	Biology and Biotechnology
Institution:	Worcester Polytechnic Institute
Title:	Assistant Professor
Signature:	
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Worcester Polytechnic Institute
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Worcester, MA 01609-2280
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).