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## Use of Drosophila S2 Cells for Live Imaging of Cell Division

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May 10, 2019

Dear Dr. Bajaj,

Please find enclosed the second revision of our article "Use of *Drosophila* S2 Cells for Live Imaging of Cell Division". We have addressed each of the editorial comments raised. Most of these have resulted in minor changes in the manuscript text, although some have been addressed with rebuttal comments expressing our decision to leave them unchanged. In all cases, the document has been edited using 'Track Changes' as requested. Below are details and justifications for these decisions:

- We have added additional details on how we typically calculate NEBD-anaphase onset time. Regarding details about analyzing mitotic spindle assembly and chromosome dynamics, we have not added these details. This is not the focus of the representative results presented. Furthermore, these additional uses of the protocol will certainly vary widely from user to user depending on the specific goals of their experiment. As such, it would not be feasible to describe all such details.
- We have added additional details to the Protocol section where prompted, including more details regarding imaging setup in the software package in order to more clearly describe **how** each step is executed. Clarifications to several steps have also been added where prompted.
- Links to the *Drosophila* resource centers were in response to reviewer suggestions. We believe they are more appropriate to leave as is rather than moving them to the references section.
- We have clarified the statements regarding dsRNA treatment and removed the NOTE detailing dsRNA amplification and primer design.
- We have added a statement in the Introduction about the copper-inducible nature of the protocol as requested.
- Finally, as requested, the Protocol sections highlighted in yellow are those we would envision being addressed in the video+commentary.

We hope you agree that we have sufficiently fulfilled the editorial requests and that the manuscript is now ready to proceed. Should you need any further information or clarification please do not hesitate to contact me.

Sincerely,

A handwritten signature in blue ink, appearing to read 'C. Johnston', is positioned in the upper left corner of the page.

Christopher A. Johnston

**TITLE:**

Use of *Drosophila* S2 Cells for Live Imaging of Cell Division

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**KEYWORDS:**

*Drosophila*, S2 cells, Mitosis, Cell cycle, Live-cell imaging, Mitotic spindle, Chromosome dynamics, mCherry: $\alpha$ -tubulin, GFP:CENP-A, CID

**SUMMARY:**

Cell divisions can be visualized in real time using fluorescently tagged proteins and time-lapse microscopy. Using the protocol presented here, users can analyze cell division timing dynamics, mitotic spindle assembly, and chromosome congression and segregation. Defects in these events following RNA interference (RNAi)-mediated gene knockdown can be assessed and quantified.

**ABSTRACT:**

*Drosophila* S2 cells are an important tool in studying mitosis in tissue culture, providing molecular insights into this fundamental cellular process in a rapid and high-throughput manner. S2 cells have proven amenable to both fixed- and live-cell imaging applications. Notably, live-cell imaging can yield valuable information about how loss or knockdown of a gene can affect the kinetics and dynamics of key events during cell division, including mitotic spindle assembly, chromosome congression, and segregation, as well as overall cell cycle timing. Here we utilize S2 cells stably transfected with fluorescently tagged mCherry: $\alpha$ -tubulin to mark the mitotic spindle and GFP:CENP-A (referred to as 'CID' gene in *Drosophila*) to mark the centromere to analyze the effects of key mitotic genes on the timing of cell divisions, from prophase (specifically at Nuclear Envelope Breakdown; NEBD) to the onset of anaphase. This imaging protocol also allows for the visualization of the spindle microtubule and chromosome dynamics throughout mitosis. Herein, we aim to provide a simple yet comprehensive protocol that will allow readers to easily adapt S2 cells for live imaging experiments. Results obtained from such experiments should expand our understanding of genes involved in the cell division by defining their role in several simultaneous and dynamic events. Observations made in this cell culture system can be validated and further investigated in vivo using the impressive toolkit of genetic approaches in flies.

## INTRODUCTION:

Cell Division is a process critical to all multicellular organisms, both in their development and homeostasis<sup>1</sup>. *Drosophila* has long been used as a model for the study of cell division, with experiments in various tissue types and genetic conditions providing key insights into the process. While many of these insights come from fixed cell conditions, cell division is a dynamic procedure with many moving parts, making visualization of live cells integral to assessing RNAi or genetic knockout effects on numerous parts of cell division, including spindle formation, chromosome congressional and segregation, and cytokinesis.

Many protocols have been developed and utilized over the years to visualize *Drosophila* cell divisions in vivo. Various groups have cultivated techniques to image divisions in both larval imaginal discs and larval brains<sup>2-8</sup>. These techniques, while useful for imaging in specific tissues, are limited in throughput and often require generation and maintenance of genetic stocks to generate fluorescent components and alter the expression of genes of interest. Cultured S2 cells from *Drosophila* provide a higher throughput alternative to rapidly test the effects of various genes in cell division. Furthermore, with the ability to transfect various fluorescent proteins, S2 cells can be quickly modified to determine the effects of RNAi knockdown on numerous components of cell division. Fluorescently tagged genes of interest can also be observed in cell division, allowing for dynamic characterization of their function<sup>9</sup>.

Here we provide a detailed protocol for live imaging of mitotic S2 cells using methods we recently described<sup>10</sup>. Our method utilizes stably transfected cells with fluorescent markers for microtubules and centromeres whose expression are under control of a copper-inducible promoter (metallothionein; pMT). This methodology can be used to image spindle and chromosome dynamics in all phases of mitosis utilizing a relatively simple fluorescent microscope with basic imaging software. It can be further customized to fit individual research needs, with transient transfection and RNAi offering expanded possibilities for determining the role of candidate genes in mitosis. Because of the relative simplicity of the protocol, it can be used for small-scale loss-of-function screens to identify genes for which further study in vivo would be beneficial, allowing for more focused efforts and efficiency with subsequent genetic manipulation in flies.

## PROTOCOL:

### 1. Preparation of S2 Cells for treatment with RNAi

1.1 From a stock culture of confluent pMT:GFP:CID and pMT:mCherry: $\alpha$ -tubulin stably transfected S2 cells (~80% viable; grown at 24-28 °C), seed cells in a 6 well sterile culture plate at a density of  $1 \times 10^6$  cells/mL in fresh, warmed, 10% fetal bovine serum (FBS) supplemented Schneider's insect media (SIM).

NOTE: Stably transfected S2 cells can be generated by following a protocol from the *Drosophila* RNAi Screening Center (DRSC) (<https://dgrc.bio.indiana.edu/Protocols?tab=cells>). Although the specific stable S2 line used herein utilizes GFP and mCherry, numerous alternatives exist both

within these fluorescent spectra as well as others. A detailed discussion of these fluorescent proteins is beyond the scope of this protocol, but their properties and potential advantages/disadvantages have been expertly reviewed elsewhere <sup>11</sup>.

1.1.1. Using a cell counter or hemocytometer, determine the density of confluent cell stock.

1.1.2. Determine the volume of confluent cell suspension necessary to achieve  $1 \times 10^6$  cells/mL in a total volume of 4 mL (e.g.,  $\sim 4 \times 10^6$  total cells). Add this volume of cell stock to a volume of fresh SIM to make 4 mL/well total volume. Determine the density of the cell stock (cells/mL) by diluting 100  $\mu$ L of cells directly from the stock flask with 100  $\mu$ L of media and counting this 1:2 dilution either manually with a hemocytometer or with an automatic cell counter if available.

NOTE: If using S2 cells not stably transfected, a transient transfection assay with fluorescently tagged (GFP, mCherry, etc.)  $\alpha$ - or  $\beta$ -tubulin, and a DNA marker (CENP-A, Histone H2B, etc.) can be performed. Further, stably transfected cell lines with various mitotic spindle and DNA markers are available from the *Drosophila* Genetics Resource Center that may better suit the exact experimental needs of the user (<https://dgrc.bio.indiana.edu/cells/Catalog>).

1.1.2. Place freshly seeded cells into 24-28 °C incubator for 36-48 h.

## 2. Treatment of seeded cells with dsRNA against gene(s) of interest

NOTE: The following example and Results section will use Shortstop (Shot), an actin-microtubule crosslinking agent as the gene of interest. Use a mock treatment with no dsRNA or with dsRNA directed against an irrelevant gene (e.g., *beta*-galactosidase, *lacZ*) as a negative control.

2.1. Warm Schneider's insect media not supplemented with FBS (Serum free media; SFM) in 24-28 °C incubator.

2.2. Transfer cells from the previously seeded well (from step 1.1.2) into a 15 mL sterile tube, noting the total volume of cells transferred.

2.2.1. Retain a small volume of cells ( $\sim 100 \mu$ L) for counting in a cell counter or hemocytometer.

2.3 Gently pellet cells by centrifuging at  $1,000 \times g$  for 3 min at room temperature.

2.3.1. While centrifuging cells, determine the concentration of cells per mL using a cell counter or hemocytometer. Multiply this number by the total volume being centrifuged (noted in 1.2.2.) to obtain the total number of cells.

2.4. Aspirate the supernatant from the pelleted cells.

2.5. Re-suspend the cells in warmed SFM to obtain a concentration of  $3 \times 10^6$  cells/mL.

2.5.1. Transfer 1 mL of the resuspended cells to a new well in a 6 well plate.

2.6. Add 10-50 µg of dsRNA (diluted in 100 µL of RNAase-free water) against Shot (or the target gene of interest) directly to the cells and swirl the plate to mix. Incubate the plate for 1 h in the 24-28 °C incubator to allow for direct dsRNA uptake into cells.

2.7. During the 1 h incubation, warm the 10% FBS supplemented SIM in the 24-28 °C incubator.

2.8. After incubating cells with dsRNA for 1 h, add 2 mL of 10 % FBS supplemented SIM directly to the well without removing the 1 mL of media.

2.9. Place cells into 24-28 °C incubator for 3-7 days.

NOTE: As with dsRNA concentration, total treatment time may need to be optimized for the desired target gene. To evaluate RNAi efficacy, perform a western blot on the whole cell lysates using an antibody against the target gene. If the initial dsRNA target sequence proves ineffective, designing alternative dsRNAs targeted to unique sequences within the target gene.

### **3. Induction of Fluorescent Protein Expression and Preparation of Cells for Imaging**

3.1. If stably transfected cells were generated using the pMT plasmid (containing an inducible metallothionein promotor) as described here, induce expression of fluorescent proteins by treating cells with copper sulfate (CuSO<sub>4</sub>) at a final concentration of 500 µM for 24-36 h prior to imaging and 4 days after RNAi treatment. Use of constitutive expression plasmids such as pAct does not require copper induction.

#### **3.2. Prepare Cells for Imaging**

3.2.1. Warm the 10% FBS supplemented SIM in the 24-28 °C incubator for 1 h.

3.2.2. Transfer cells into a 15 mL sterile tube, noting the total volume of cells transferred.

3.2.2.1. Retain a small volume of cells (~100 µL) for counting in a cell counter or hemocytometer.

3.2.3. Gently pellet cells by centrifuging at 1000 x g for 3 min at room temperature.

3.2.3.1. While centrifuging cells, determine the concentration (cells/mL) using a cell counter or hemocytometer. Multiply this number by the total volume being centrifuged (noted in 2.2.2.) to obtain the total number of cells.

3.2.4. Aspirate the supernatant from the pelleted cells.

3.2.5. Resuspend the cells in fresh, warmed 10% FBS supplemented SIM to obtain a concentration of  $2 \times 10^6$  cells/mL. Add an appropriate amount of  $\text{CuSO}_4$  to maintain 500  $\mu\text{M}$  concentration.

3.2.6. Transfer 200-500  $\mu\text{L}$  of re-suspended cells to one well of a multi-well live cell chamber and place on the inverted fluorescent microscope. Allow the cells to settle in the chamber for 15-30 min prior to imaging experiments.

NOTE: Live cell chamber wells can be pre-coated with poly-L-lysine for additional adherence.

#### 4. Set up a live cell imaging program

NOTE: Live Cell Imaging in this experiment was performed using an inverted imaging system and its associated software (e.g., Olympus IX83 with the cellSens Dimensions software package). Details will differ by microscope manufacturer and software package; thus, general guidelines and operations are listed below.

4.1. Using the software that runs the inverted fluorescent microscope, prepare a program for imaging a cell (or cells) over time.

4.1.1. Open the software by double-clicking on the software desktop icon.

4.1.2. Create a new experimental file by clicking **File**, then **New experimental file**.

4.1.3. First, insert a time lapse loop over which images will be taken. Do this by clicking the **Stopwatch** icon (**Time-lapse Loop** icon) from the icon bar. Set the interval in s in the **Experiment Manager** tab, and then set the number of cycles in the same tab by dividing the desired overall experiment length (in s) by the interval. Allow the loop to repeat over the desired total time period.

NOTE: Typically, images are taken every 30-60 s over a period of 3-4 h.

4.1.4. Within the time lapse loop layer, insert an infrared focus check to maintain the focus of the objective (if available). To do this, add a Z-drift Compensation (ZDC) step within the Time-lapse Loop layer by clicking on the **Square with Two Arrows** icon (Move XY icon) and selecting **Z-Drift Compensation** from the dropdown menu.

NOTE: The term infrared focus check refers to a system by which an infrared pulse is used to maintain a constant distance between the objective and the slide/imaging chamber. Many microscopes have such a system, each with their own proprietary nomenclature. Readers should consult their operation manual or representative for specific naming details.

4.1.5. After the infrared focus check, add a step in the program to take a multichannel image (e.g., FITC for GFP and TRITC for mCherry) over a 3-5 z-stacks by first inserting a z stack step,

then specifying the channel. Do this by adding a Multichannel Group layer within the Time-lapse loop layer by clicking on the **Color Wheel** icon (**Multichannel Group** icon). Next, add a Z-Stack Loop layer within the Multichannel Group layer by clicking on the **3-layered icon** (**Z-stack loop** icon). Set the desired Step Size and Number of Slices in the **Experiment Manager** tab and set the exposure of each channel as low as possible to minimize photobleaching.

NOTE: The z-stack interval, exposure time, and percent transmittance for LEDs can vary. Typical in these experiments, use three z-stacks taken over a range of 3  $\mu\text{m}$ , giving an interval of 1  $\mu\text{m}$  between each stack. Defining the center of the cell rather than the top and bottom tends to lead to the best results. Images are then collected for each channel(s) at an exposure of 50 ms and percent transmittance of 50% with no neutral density (ND) filter engaged.

## 5. Image Dividing Cells Using Live Cell Imaging Program.

NOTE: S2 cells do not require  $\text{CO}_2$  and grow optimally at 23-27  $^{\circ}\text{C}$ . All imaging was performed at ambient temperature in a well-controlled room.

5.1. Using the oculars, find the top (or bottom) corner of the well and focus the objective (40-60x oil immersion) onto the cells using the mCherry ( $\alpha$ -tubulin) channel.

5.2. Scan along the top (or bottom) of the well to move away from the vertical well divider.

NOTE: Imaging too close to the well dividers can interfere with infrared focus checks.

5.3. Find a cell (or cells) that are in late G2 or early M-phase (prophase).

NOTE: These cells can be best identified by the existence of exactly two 'star-like' microtubule structures (centrosomes) and an intact nucleus (indicated by a circular region of refracted light within the cell), both easily distinguished using the  $\alpha$ -tubulin (red) excitation filter. Selection of cells in earlier interphase, notable by one or zero easily visible centrosomes, can result in wasted time due to a lag in G2/M progression. Conversely, selection of cells after NEBD will prevent accurate calculation of mitotic timing and imaging of early spindle assembly and chromosome dynamics. Also, S2 cells often contain >2 centrosomes. Although these typically clusters into a bipolar spindle<sup>12</sup>, it is advised that users avoiding these cells unless otherwise desired for the experimental design. Images and discussion of appropriate cells to select can be found in the **Representative Results** section.

5.5. Click the **Live** view button to begin viewing cells in the software screen. Using the fine focus knob of the microscope, focus the cell (or cells) of interest. Set the infrared focus check by clicking the **Set Focus** button.

5.6. Initiate the time lapse imaging program by clicking the **Start** button. Adjust the histograms as needed by selecting the desired channel and adjusting the mean pixel intensities to see the cell (or cells) of interest clearly.

5.7. Allow the program to run, checking the cell after 15-20 min to ensure nuclear envelope breakdown (NEBD) has occurred, which is determined by the disappearance of the round dark, a refracted spot near the cell center.

5.8. Continue allowing the program to run, checking intermittently (every 15-20 min) to determine once anaphase onset has occurred. Stop the program at this point if there is more time remaining in the total time period and save the file. Alternatively, if events during telophase and cytokinesis are of interest, allow the program to run a sufficient time in order to image these processes as well.

5.9. Perform analysis of NEBD to anaphase onset timing by noting the time of NEBD ( $t_{\text{NEBD}}$ ) in min and the time of initial chromosome separation ( $t_{\text{anaphase onset}}$ ) in min and subtracting  $t_{\text{NEBD}}$  from  $t_{\text{anaphase onset}}$  to obtain the NEBD to anaphase onset time for a given cell.

5.9.1. Do this by clicking the **frame up** button and noting the frames where NEBD and anaphase onset occur, subtracting these to determine the total number of elapse frames, and multiplying this by the time interval between imaging frames.

5.10. Continue scanning for pre-dividing cells to obtain multiple n's for a given condition. Cells can be imaged in the live cell chamber well for up to 12 h after their initial settling.

## REPRESENTATIVE RESULTS:

Methods we describe above will result in the identification and imaging of *Drosophila* S2 cells undergoing cell division. Cells that are about to divide (i.e., just prior to entering M-phase) can be targeted by the presence of two centrosomes and an intact nucleus indicated by refracted light and a darker spot within the cell when viewed in the  $\alpha$ -tubulin channel (**Figure 1**, left-most panels, red channel; arrowhead in **Figure 1A**). We estimate that approximately 2-5% of cells fall into this category, and between imaging experiments, we typically spend a maximum of 2-3 minutes scanning for another qualified cell. NEBD can be visualized through the disappearance of this dark spot, resulting in the uniform coloring of the cytoplasm (**Figure 1**, panels marked "00:00", red). After NEBD, the time each cell takes to form the spindle, congress chromosomes, and segregate chromosomes can be measured simply by noting the time points of these events relative to NEBD.

We utilize these divisions to assess mitotic timing, specifically of NEBD to anaphase onset, noting the point at which chromosomes begin to separate. A majority (~90%) of copper-induced cells, expressed both mCherry: $\alpha$ -tubulin and GFP:CID. A small percentage of cells (~10%) expressed only one marker or neither. These cells were avoided during cell selection. Typically, S2 cells display NEBD to anaphase onset timing of between 20-30 minutes (**Figure 1A**, Control). We next treated cells with dsRNA targeted against Shortstop (Shot), an actin-microtubule crosslinking protein we suspected may affect cell cycle dynamics. Indeed, following Shot knockdown cells exhibited a significant mitotic delay (**Figure 1B**, ShotRNAi delay), with many cells arresting at metaphase and never transitioning to anaphase during the entire 2-3-

hour imaging experiment (**Figures 1D**, ShotRNAi arrest). We reasoned this delay/arrest phenotype could likely be due to activation of the spindle assembly checkpoint (i.e. the M-phase checkpoint). To directly test this hypothesis, we co-treated cells with dsRNA against Shot and Rough deal (Rod), an important component of this checkpoint<sup>13</sup>. This led to a suppression of the arrest phenotype, resulting in NEBD to anaphase times similar to Control (**Figure 1C**, ShotRNAi+RodRNAi). Thus, this live imaging protocol allowed us to conclude that Shot is required for timely M-phase progression and that its loss leads to checkpoint activation that delays or arrests mitotic cells.

#### FIGURE LEGENDS:

**Figure 1: Live imaging reveals cell cycle defects due to mitotic checkpoint activation in S2 cells.** In all conditions, live-cell imaging was conducted on S2 cells stably co-transfected with inducible GFP:CID and mCherry:αTubulin as described herein. (**A**) Cartoons illustrate important landmarks during mitosis, and corresponding images are shown from representative movies of indicated genotypes with time points relative to NEBD (t=00:00) indicated. Control cells typically progress to anaphase within 20-30 min. ShotRNAi-treated cells display NEBD-anaphase delay (**B**) and frequently undergo metaphase arrest, never entering anaphase within the imaging experiment (**D**). Co-treatment of cells with ShotRNAi and RodRNAi, a component of the spindle assembly checkpoint, suppresses the Shot phenotype leading to anaphase onset kinetics similar to Control cells (**C**). The arrows in (**A**) indicate the ‘star-like’ centrosome structures, and the large arrowhead indicates the nucleus. Each scale bar represents 5 microns. This figure is adapted and republished with permission from<sup>10</sup> (<https://www.molbiolcell.org/info-for-authors>).

**Video 1: Time-lapse movie of ‘Control’ S2 cell division.** Video shows a representative movie of S2 cell division in ‘Control’ genotype. This movie corresponds to **Figure 1A**. This video is adapted and republished with permission from<sup>10</sup> (<https://www.molbiolcell.org/info-for-authors>).

**Video 2: Time-lapse movie of delayed ‘ShotRNAi’ S2 cell division.** Video shows a representative movie of S2 cell division in ‘ShotRNAi’ genotype that leads to a phenotypic mitotic delay. This movie corresponds to **Figure 1B**.

**Video 3: Time-lapse movie of ‘ShotRNAi+RodRNAi’ S2 cell division.** Video shows a representative movie of S2 cell division in ‘ShotRNAi+RodRNAi’ genotype. This movie corresponds to **Figure 1C**. This video is adapted and republished with permission from<sup>10</sup> (<https://www.molbiolcell.org/info-for-authors>).

**Video 4: Time-lapse movie of arrested ‘ShotRNAi’ S2 cell division.** Video shows a representative movie of S2 cell division in ‘ShotRNAi’ genotype that leads to a phenotypic mitotic arrest. This movie corresponds to **Figure 1D**.

#### DISCUSSION:

### **Identifying an Appropriate Cell**

Key to imaging dividing S2 cells is first locating the proper cell. Time can be wasted imaging cells that are mistakenly thought to be ready to divide but fail to do so in a reasonable timeframe. Cells must be identified that have two distinct and visible centrosomes and an intact nucleus. Centrosomes must have microtubule fibers emanating from them, giving them a “star-like” appearance. An intact nucleus refracts light when adjusting focus, and also makes the approximate center of the cell darker in appearance. The nucleus will also contain the GFP::CID “dots”, another distinguishing feature to help in its identification. Cells with >2 centrosomes, tubulin puncta without tubulin fibers emanating from them, or cells with only one centrosome should be avoided. Additionally, if two centrosomes are visible, but a nucleus is not, NEBD has already occurred and the cell is too far advanced in its division to be imaged if a complete M-phase analysis is desired. Once an appropriate cell is found, speed in starting imaging is crucial as NEBD occurs quickly (typically within 3-5 min) and delaying the start of imaging can lead to missed opportunities. Focus the cell in the imaging software quickly such that both centrosomes are visible, or if centrosomes are out of plane with each other, set the focal point between the two, such that each can be captured when Z stacks are collected above and below the defined center point. Once focused, immediately set the offset between the stage and the live cell chamber surface using the infrared focus check (if available) and begin the imaging program. Although the protocol presented here is specifically tailored for experiments assessing NEBD-to-anaphase dynamics, simple modifications could suit readers studying alternative mitotic events. For NEBD-to-metaphase and anaphase-to-telophase imaging, we suggest 10 s image capture intervals as these processes are highly dynamic and occur quickly in S2 cells (5-10 min). Metaphase-to-anaphase transition (our typical experimental focus) is a longer process (20-30 min) in S2 cells, and we collect images at 30 s intervals. Lastly, telophase-through-cytokinesis occurs quite slowly in S2 cells, and we suggest 60 s intervals providing enough resolution for this approximate hour-long process.

### **Maintaining Focus Throughout the Imaging Program**

If an infrared focus check device is not available, be sure to avoid bumping the microscope or table it sits upon, as this can result in the cell moving out of focus during the imaging program, leading to ambiguous, un-interpretable results. Pre-coating the live cell chamber wells with Poly-L-lysine can help in cell adherence, which can help avoid cell movement and is especially useful for setups without infrared focus checks. Additionally, setups including shock-absorbing platforms or air tables can further help avoid cells moving out of focus. Lastly, some software programs have pause functions allowing the user to refocus and resume the program.

### **Imaging Multiple Cells**

Helpful to accumulation of data is that often multiple cells ready to divide can be placed within the same field of view for imaging. This can be especially useful for experiments in which cells have long M-phase durations or prolonged arrest (e.g., conditions that induce activation of the SAC). For these cells, we typically image until the cells are photobleached (approximately 2-3 h), but the full timing of arrested cells should be at the discretion of the researcher. Sometimes the multiple pre-dividing cells can be in different focal planes, in which case it may be useful to add z-stacks to accommodate all cells. Adding more z stacks can also help improve resolution.

Caution should be taken in doing this, however, as it will expose cells to more radiation and lead to quicker photobleaching, as well as lead to large file sizes on hard disk storage devices.

### **Avoiding Photobleaching and Phototoxicity**

Our method describes specific settings for exposure times, imaging intervals, z stacks, and percent transmittance for our LED light source that generally work for our experimental setup and desired outcomes. As microscopes and experimental goals may vary, what might work well for our system may lead to premature photobleaching or phototoxicity in others. Photodamage may present a lack of spindle movement, microtubule fragmentation, defective microtubule dynamics, and prolonged spindle checkpoint activation. One potential method to avoid such pitfalls is to limit the exposure time. Most modern cameras now possess wide dynamic ranges, and histograms can be adjusted to visualize structures even at very low exposures. Another technique is to increase the imaging interval (e.g., to several minutes between images) such that the number of times a cell is exposed to light over a total collection interval is reduced. This is advantageous particularly in imaging for longer periods of time (many hours), and can additionally help in decreasing the file size of such experiments. Limiting the number of z stacks taken can also help reduce light exposure, using 2 or even just 1 instead of 3. Further, adjusting the percent transmittance of light (for LED light sources), or utilizing neutral density (ND) filters (for both Halogen Lamp and LED light sources) will decrease the light intensity and coupled with longer exposure times can preserve visibility. By choosing cells with centrosomes already separated, overall exposure can be limited in that these cells usually enter mitosis quickly (within a minute or two) after beginning imaging. One can also limit the time that cells are allowed to be imaged before NEBD. Our lab typically sets this time at 10 min, but a more conservative limit can easily be imposed by the user. Additionally, a more 'invasive' measure we recommend is treatment with dsRNA targeted against a component of the SAC (such as Rod or Mad2). If an arrest phenotype is due to non-specific cell damage (e.g., defective microtubule dynamics), such a treatment is less likely to suppress the arrest compared to a *bona fide* effect of the gene of interest in the original experiment.

### **Future Directions**

The method described here can be utilized on a relatively simple epifluorescent microscope to rapidly image live cell divisions and can be easily adapted to suit a researcher's particular experimental design and goals. Several excellent methods for culturing, RNAi knockdown approaches, transient transfection, and fluorescence microscopy have been published for S2 and other *Drosophila* cells<sup>9,14-17</sup>. Our protocol offers a couple of advantages. (1) The use of a double stable cell line (GFP:CID, mCherry:α-Tubulin) simultaneously marks two key mitotic structures, avoids the hassle and potential complications of transient transfection, and ensures that nearly all cells will express fluorescent markers as potential candidates for imaging. (2) The adaptation to mitosis adds to published protocols examining cytoskeletal dynamics in motile, non-dividing cells and extends to examining mitotic events in real time. While we believe ours is a powerful technique that adds to the repertoire of others, there can always be improvements made. One particular area of cell division that we have found hard to image is centrosome division. This occurs prior to NEBD and it is difficult to determine when a single centrosome is ready to separate into two. Using fluorescent cell cycle markers (Cyclin A and Cyclin B) could

help resolve this problem, but comes at the expense of channels that could be used to visualize cell division components. Our best strategy for attempting to visualize centrosome division is to add multipoint acquisition to the imaging program (defining different points in the XY plane to image), but this can result in large files, and can also require (depending on the number of points) increasing the interval between image collection, decreasing time resolution of the resulting movie. Another solution could be synchronization of cells at a desired cell cycle stage using drugs that target cell cycle regulators followed by subsequent washout prior to imaging, although these methods may not be reliable in S2 cells specifically.

The protocol presented here provides a foundation for future applications in live cell imaging as well. With improved imaging and software technology, truly high throughput adaptations of this protocol using higher capacity, multi-chambered plates could be possible. Such innovations would make large-scale RNAi screens, such as those already achieved in fixed preparations<sup>12,18</sup>, more tractable in a live cell format. Small molecule drug screens could also be envisioned as a means of identifying novel compounds targeting cell division processes. Improvement of fluorophores and optical filters could also lead to imaging of multiple mitotic components (not just the two we describe), enabling imaging of specific mitotic regulators and their interactions with DNA and/or the spindle. For example, generating cells with fluorescent reporters that express following the DNA damage or during apoptosis would be useful tools to identify novel genes involved in these processes. Similar approaches could be used to examine cell cycle dynamics using the expression of fluorescently tagged cyclins<sup>19</sup>.

#### ACKNOWLEDGMENTS:

This work was funded by the National Institutes of Health (R01 GM108756). We are grateful to Gary Karpen (University of California, Berkeley) for generously providing us with a GFP:CID S2 cell line stock from which our GFP:CID/mCherry:αTubulin line was generated<sup>10</sup>.

#### DISCLOSURES:

The authors have nothing to declare.

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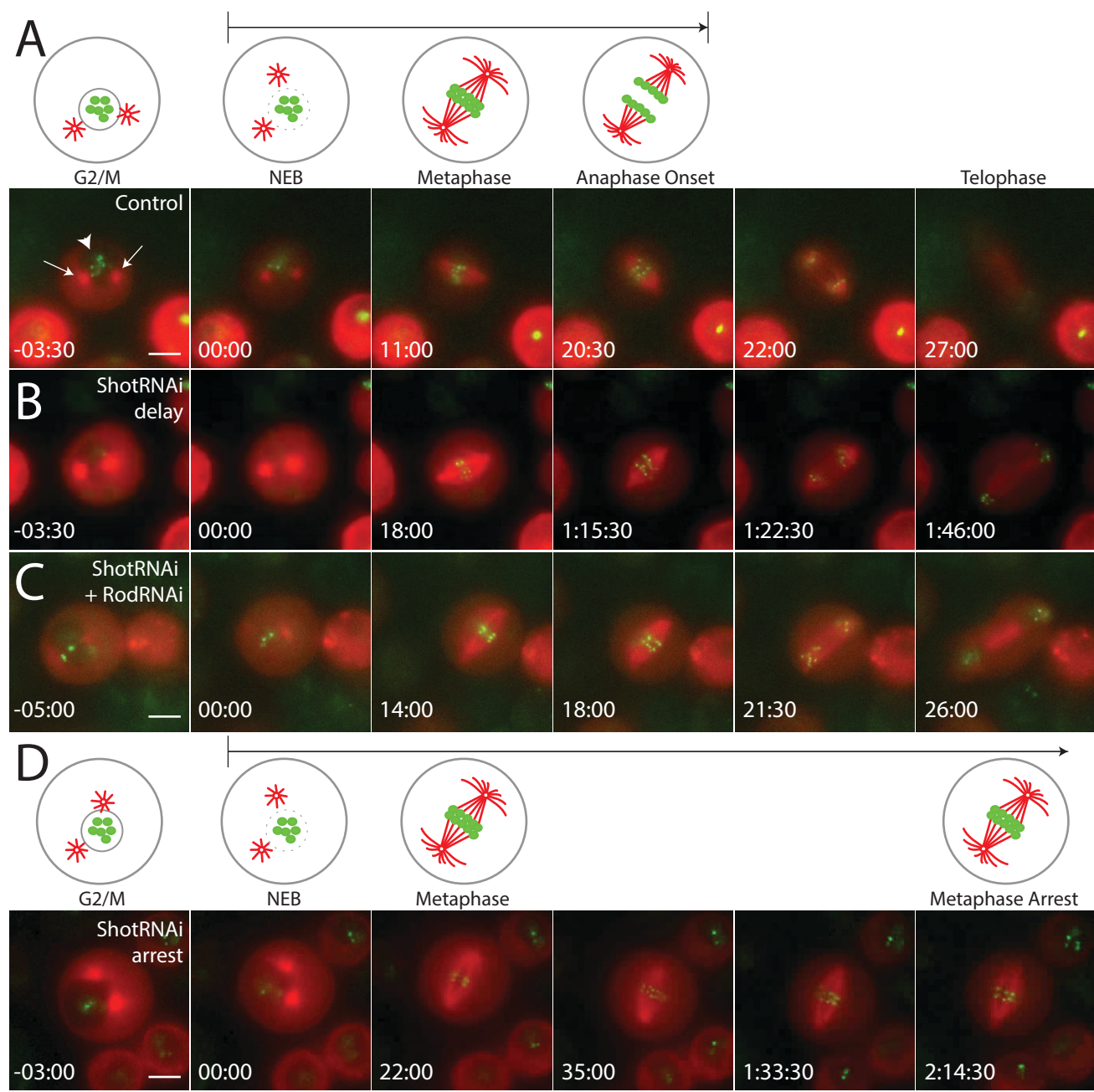
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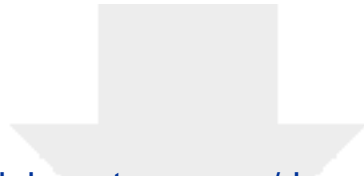
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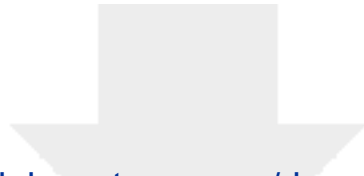


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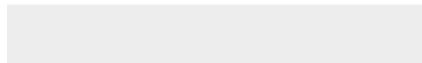


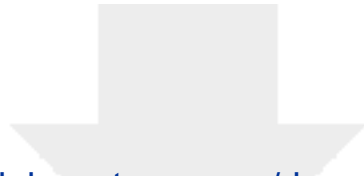


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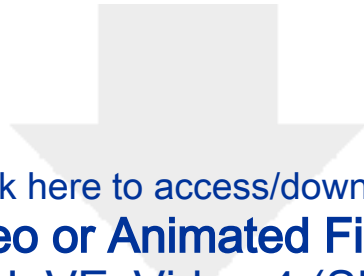


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| Name of Material/ Equipment  | Company                           | Catalog Number | Comments/Description       |
|--|-----------------------------------|----------------|----------------------------|
| Bright-Line Hemacytometer  | Sigma-Aldrich                     | Z359629-1EA    | for cell counting          |
| cellSens imaging software  | Olympus                           |                |                            |
| CELLSTAR Cell Culture Flask,<br>50ML, 25 CM2, PS, Red Filter<br>Screw Cap, Clear, Sterile,<br>10PCS/BAG                    | Greiner Bio-One                   | 690175         |                            |
| CELLSTAR Cell Culture Multiwell<br>Plate, 6 well, PS, Clear, TC, Lid<br>with condensation rings, sterile,<br>single packed | Greiner Bio-One                   | 657160         |                            |
| Centrifuge 5804 R  | eppendorf                         | Cat. 022623508 |                            |
| Copper(II) sulfate pentahydrate,<br>minimum 98%  | Sigma-Aldrich                     | C3036-250G     |                            |
| Corning Fetal Bovine Serum   | Fisher Scientific                 | MT35015CV      |                            |
| Effectene Transfection Reagent 1<br>mL   | Qiagen                            | 301425         | for transient transfection |
| IX-83 Inverted Epifluorescent<br>Microscope  | Olympus<br>Applied Scientific     |                |                            |
| LabTek Chambered Slide Insert  | Instruments<br>Thermo Fisher      | I-3016         |                            |
| MEGAscript T7 Transcription Kit  | Scientific                        | AM1334         | For dsRNA production       |
| MOXI Z Mini Automated Cell<br>Counter Kit  | ORFLO                             | MXZ001         | for cell counting          |
| MS-2000 XY Flat-Top Automated<br>Stage and Controller  | Applied Scientific<br>Instruments |                |                            |
| Nunc Lab-Tek II Chambered<br>Coverglass (no 1.5 borosilicate<br>glass) 8-well  | Thermo Fisher<br>Scientific       | 155409         |                            |

|                                   |                       |             |                      |
|-----------------------------------|-----------------------|-------------|----------------------|
| Orca-Flash 4.0 LT Camera          | Hamamatsu             |             |                      |
| pMT/V5-His A <i>Drosophila</i>    | Photonics K.K.        | C11440-42U  |                      |
| Expression Vector                 | Thermo Fisher         |             |                      |
| Poly-L-Lysine                     | Scientific            | V412020     |                      |
| Purifier Logic+ Class II, Type A2 | Cultrex               | 3438-100-01 |                      |
| Biosafety Cabinets                | Labconco              | 302310000   |                      |
| Schneider's insect medium         | Sigma-Aldrich         | S0146-100ML |                      |
| Spectra Tub Centrifuge Tubes      | VWR                   | 470224-998  |                      |
|                                   |                       |             |                      |
| Uner Counter BOD Incubator        | Sheldon               |             |                      |
|                                   | manufacturing (VWR)   | 89409-346   |                      |
|                                   |                       |             |                      |
| X-CITE 120 LED                    | ExcelitasTechnologies |             | Led light source     |
| Z -Drift Compensator (ZDC)        | Olympus               |             | infrared focus check |



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May 6, 2019

Dear Dr. Bajaj,

We are pleased to submit this revised version of our previously reviewed manuscript entitled “Use of *Drosophila* S2 Cells for Live Imaging of Cell Division” to *JoVE* for your consideration for publication.

We have made all formatting changes requested in the Editorial Comments, apart from the journal title formatting of the references (comment #13). We use EndNote and downloaded the Style file directly from the link provided in the ‘Instructions to Authors’. If you would like them changed/expanded, please inform us as to which specific EndNote Style we should use.

We sincerely appreciate the reviewer comments and their collective efforts to improve the quality of our manuscript. We have, to the best of our ability, addressed the concerns raised by each reviewer. Below we detail our responses, including indicating the changes to the manuscript where relevant, to each reviewer comment. We are confident we have satisfied each concern and hope the reviewers and editorial staff agree that our revised manuscript is a stronger presentation of the protocol.

#### **Reviewer #1:**

1. The reviewer’s suggestion to including methods for S2 cell transfection and generating stable cell lines certainly has merit. Although we agree it would be of potential benefit to some readers, this is unfortunately not the focus of the manuscript and discussions with editorial team explicitly instructed us not to focus on this aspect. As a compromise, we have added a short, general statement about stable cell line generation and provide references to excellent resources for additional details (Lines 95-100). We also provide information and a link to the DGRC as the reviewer suggests (Lines 122-124).
2. We have now added a “NOTE” line (lines 199-201) to direct readers to western blotting as the preferred technique for assessing RNAi efficacy. We have also added a statement that suggests readers try alternative transcript targets (e.g. alternative RNAi amplicons) if their first fails. We add a link to the Harvard SnapDragon web-based design engine as our preferred method of RNAi target and primer design (Lines 201-205).

We have added a suggestion to decrease image acquisition frequency or number of z-stacks taken as alternatives to reduce photobleaching (Lines 417-421).

Finally, we add a new section in the Discussion, 'Avoiding Photobleaching and Phototoxicity', specifically referencing the signs of damage suggested by the reviewer (lines 779-804). We suggest that if these phenotypes are seen, the reader consider reducing image exposure time, frequency, and/or z-stack number. Regarding mitotic arrest, in order to differentiate between photo-damage a *bona fide* phenotype of RNAi treatment, we suggest that readers perform a co-RNAi treatment against components of the Spindle Assembly Checkpoint (e.g. Rod, Mad2). If such treatment suppresses the arrest phenotype, this can add support to it being due to the original RNAi treatment rather than general photo-damage.

- Finally, videos and still-frame images presented are cropped from original files to focus on individual cells. However, the 60x objective used for actual imaging captures many (~100) cells within the field of view for any given experiment. As a result, we often experience multiple mitotic cells within one imaging experiment. Notably, in many cases we will witness one fully-arrested cell along with another cell that, while mitotically delayed, still divides. This cell that completes mitosis was, however, subjected to the same photoexposure dose as the arresting cell. This result is more consistent with the arresting phenotypes being due to a molecular effect of the dsRNA treatment rather than phototoxicity. As this argument is admittedly nuanced, we have not included this discussion in the manuscript, although we would be happy to if the editorial team thinks it is warranted.

#### **Reviewer #2:**

1. We have added details about the typical exposure times for GFP and mCherry (Lines 420-421).
2. We have added details about the typical times expected for progression through subphases of mitosis, specifically highlighting that S2 cells are known to be limited at the metaphase-anaphase transition (Lines 734-741).
3. We add a description of our typical percent transmittance and discuss the advantage of optimizing this against exposure time to maximize signal while minimizing photo-damage (Lines 420-421; 779-804).
4. We have clarified the "dark spot" being found in the tubulin (mCherry) channel (Lines 438-446). We have also added an arrow to the first still frame image in the figure to clearly indicate its appearance to readers.

#### **Reviewer #3:**

(Major comment) – Along with similar concerns raised by other reviewers, we have attempted to better address the issue of photo-damage/toxicity and its potential to complicate phenotypic interpretations (e.g. cell cycle arrest due to RNAi vs. phototoxicity). We appreciate the suggestion and agree it was an oversight not to more clearly discuss the

importance of distinguishing potential artifacts of cell toxicity. We have added additional details regarding image acquisition and temperature as requested (420-421; 426-427). In the event of a mitotic arrest phenotype, we first suggest users reduce image acquisition frequency (which will lower photoexposure dosage). This is the optimal first step as it will not only reduce phototoxicity likelihood, but should not sacrifice significantly the video resolution. Other simple modifications include lowering exposure time and/or transmittance (see Reviewer 2). A more 'invasive' measure we recommend would be the treatment with RNAi targeted against a component of the spindle assembly checkpoint (e.g. Mad2 or Rod that we have successfully used). If an arrest phenotype is due to non-specific cell damage (e.g. defective microtubule dynamics), such treatment is less likely to suppress the arrest compared to a *bona fide* effect of the gene of interest in the original experiment. This method, however, should not be taken as conclusive, but rather as additional support together with simple modifications to the imaging protocol that directly reduce photoexposure. This discussion can be found on the new Lines 779-804.

1. We have replaced the old Video 2 with an alternative example (new Video 4) for what we now label as "ShotRNAi Arrest". This video is of higher quality and on par with the other videos. Still-frame images have also been updated in Figure 1 to match. Furthermore, this video is less striking in the "optication"-like effects noted by the reviewer for the original uploaded file. We have also added a new video (Video 2) and images to Figure 1 for a "ShotRNAi Delayed" phenotype wherein the cell has a significant delay in anaphase onset yet does proceed through mitosis without arrest.
2. We have now added time-stamp annotations to all videos. The text first appears at NEBD, 00:00:00, for each and continues through the end of the video.
3. We have changed the NEB acronym to NEBD throughout the manuscript as suggested.
4. We agree that the dsRNA treatment represents a new phase of the protocol worth designation as a new section. As such, the old "1.2" has been made "2.1" and numbering of all subsequent sections have been adjusted accordingly.
5. This has been fixed.
6. We have added a short description of what we mean by "infrared focus check" and indicate that specific nomenclature will differ by software manufacturer (Lines 353-356). However, we avoid using any specific term to comply with *JoVE* instructions on not using proprietary names.
7. We have now explicitly defined the Shot gene (Lines 541-542).
8. This is indeed a *JoVE* formatting issue. Thus, we have left the manuscript order as is.

**Reviewer #4:**

1. We have added a statement regarding the approximate %cells in prophase and ideal for selection (Line 531). This is also expressed in an average time scanning for an ideal cell in between each experiment (Lines 531-532).

We hope you agree that we have sufficiently fulfilled the reviewer requests. Should you need any further information or clarification please do not hesitate to contact me. My co-author and I look forward to your editorial decision and discussing the next steps in the process.

Sincerely,



Christopher A. Johnston

## Information

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