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# Title: Laser Micro-Irradiation to Study DNA Recruitment During S Phase

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# Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group?

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

**Current Protocol Length**

Number of Steps: 15
Number of Shots: 23

# Introduction

**1. Introductory Interview Statements**

**REQUIRED:**

1.1. **Bearach Miwatani-Minter:** This protocol helps to understand the spatial and temporal recruitment of DNA damage repair proteins taking into consideration the cell cycle phase.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.2.1*

1.2. **Bearach Miwatani-Minter:** For cell cycle discrimination we use fluorescent protein tagged PCNA as a marker of S-phase, which circumvents artefacts introduced by other cell cycle synchronization methods. This combined with laser based micro-irradiation gives unparalleled spatio-temporal resolution to DNA damage repair protein kinetics.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: LAB MEDIA: Figure 3A, emphasize S phase rows.*

**OPTIONAL:**

1.3. **Bearach Miwatani-Minter:** Successful and routine utilization of our protocol builds on basic knowledge of confocal imaging.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 3.2.2.*

# Protocol

## 2. Preparation of Cells for Micro-irradiation

2.1. 24 hours before the micro-irradiation **[1]**, plate a total of 8 x $10^4$ cells in 500 microliters to 1 milliliter of media on a four well chambered coverglass **[2]**.

2.1.1. WIDE: Establishing shot of talent at the biosafety cabinet, cells and coverglass in view.

2.1.2. Talent plating cells. *Videographer: This step is important!* NOTE: Shot was filmed both WIDE and CU, can switch between both as needed.

2.2. One hour before the micro-irradiation, exchange the regular growth medium with imaging medium containing either Olaparib or a vehicle control such as DMSO **[1-TXT]**.

2.2.1. Talent replacing the growth medium with imaging medium. **TEXT: See text for imaging media preparation details; Olaparib = PARP inhibitor at 1 µM final concentration**

## 3. Selecting S Phase Cells for Imaging

3.1. At least 4 hours before imaging, turn on the environmental chamber and the microscope components. Switch on the heating, carbon dioxide supply, and the humidity regulator **[1]**. Initialize light sources along with the laser lines at least 1 hour before transferring the cells to the microscope **[2]**. Oil the microscope objective and place the chambered coverglass under the microscope. *Videographer: This step is important!*

3.1.1. Talent turning on the environmental chamber and microscope components.

NOTE: This shot was split into two shots as the room was too small to get everything in view in one shot.

3.1.2. Talent initializing the light sources. NOTE: This is included in shot 3.1.1.

Added Shot: Talent oiling the objective and adding the coverglass.

NOTE: Use the shot taken from a higher angle to capture the oiling of the objective.

3.2. To select S-phase cells in an asynchronous population, look through the ocular **[1]** for the unique localization pattern of the mPlum *(M-plum)*-tagged PCNA in S phase and select a field of view that has enough S-phase cells for micro-irradiation **[2]**.

3.2.1. Talent looking through the ocular.

3.2.2. SCREEN: 62466_screenshot_1. 0:03-0:12.

3.3. Set the region of interest for micro-irradiation by using the associated software to insert binary lines **[1]**. To set the desired number of lines and spacing, click **Binary**, then select **Insert line**, **Circle**, and **Ellipse** for drawing the desired number of lines **[2]**.

3.3.1. Talent at the computer. *Videographer: Obtain a few shots of talent clicking the mouse and typing on the keyboard to use as b-roll throughout the video.*

3.3.2. SCREEN: 62466_screenshot_1. 0:14-0:23.

3.4. Then, convert these binary lines into stimulation ROIs by clicking **ROI**, **Move Binary to ROI**, then right click on any of the ROIs and select **Use as Stimulation ROI-S1** **[1]**.

3.4.1. SCREEN: 62466_screenshot_1. 0:23-0:34.

3.5. Place these lines in the field of view so that they pass through the nucleus of the cells **[1]**.

3.5.1. SCREEN: 62466_screenshot_1. 0:35-0:46, then skip to 0:52.

**4. Micro-irradiation for Immunofluorescence Staining or Time Lapse Imaging**

4.1. Before micro-irradiation, to identify PCNA foci for later analysis, take a higher resolution image of the field of view by setting the necessary parameters in the **A1 LFOV** *(L-F-O-V)* **Compact GUI** *(G-U-I)* and the **A1 LFOV Scan Area** windows followed by hitting the **Capture** button **[1]**.

4.1.1. SCREEN: 62466_screenshot_2. 0:04-0:22.

4.2. To set up the micro-irradiation, open the **ND Stimulation** tab and access the **Time schedule** window to acquire a series of pre-stimulation images and then a series of post-stimulation images using the galvano scanners **[1]**.

4.2.1. SCREEN: 62466_screenshot_3. 0:04-0:14.

4.3. Set up three phases in the **Time schedule** window **[1]**. In the **Acquistion and Stimulation** column, select **Acquisition**, **Stimulation**, and **Acquisition** for the three phases, respectively **[2]**. For the **Stimulation** phase, set **S1** as the ROI **[3]**.

4.3.1. SCREEN: 62466_screenshot_3. 0:14-0:19.

4.3.2. SCREEN: 62466_screenshot_3. 0:19-0:23.

4.3.3. SCREEN: 62466_screenshot_3. 0:23-0:27.

4.4. In the **Galvano XY window**, set 405 nanometers as the laser power output and set the dwell time for micro-irradiation **[1]**.

4.4.1. SCREEN: 62466_screenshot_4. 0:05-0:13.

**5. Time Lapse Imaging**

5.1. To set up time lapse imaging for the desired time window and intervals, use the **Time schedule**, **A1 LFOV Compact GUI**, and the **A1 LFOV Scan Area** windows **[1]**. Optimize the **laser power %**, **gain** and **offset settings** to reduce photo-bleaching during imaging in the **A1 LFOV Compact GUI** window **[2].**

5.1.1. SCREEN: 62466_screenshot_5. 0:07-0:29

5.1.2. LAB MEDIA: still image 1. *Video Editor: Show only the **A1 LFOV Compact GUI** window (top window). Emphasize the adjustable settings: HV (GaAsP), Offset and Laser setting (488 and 561) under the checked boxes for FITC and TRITC at the bottom of the window*

5.2. Depending on the kinetics of the protein, extend or shorten the interval between images or the duration of the total time lapse by setting the desired **Interval** and **Duration** for the third phase Acquisition row in the **Time Schedule** window **[1]**.

5.2.1. SCREEN: 62466_screenshot_5. 0:30-0:45.

5.3. Press **Run now** to execute the micro-irradiation and the subsequent time lapse imaging **[1]**. At the end of the time lapse imaging, save the stimulation ROIs as separate images, which will be useful for identifying the coordinates of micro-irradiation in any downstream software used for analysis **[2]**.

5.3.1. SCREEN: 62466_screenshot_6. 0:06-0:17, then skip to 0:42-0:44.

5.3.2. SCREEN: 62466_screenshot_7. 0:03-0:16.

# Results

## 6. Results: DNA Recruitment During S Phase After Micro-Irradiation

6.1. PCNA has a completely homogeneous distribution in the nucleus in G1 and G2 phases **[1]**. In S phase, PCNA localizes to sites of DNA replication, which can be visualized as bright spots in the nucleus **[2]**.

6.1.1. LAB MEDIA: Figure 1A. *Video Editor: Show either one of the two rows of panels. Emphasize the G1 and G2 panels*

6.1.2. LAB MEDIA: Figure 1A. *Emphasize the S panel in the same row*

6.2. In early S phase cells, the spots are relatively small and equally distributed throughout the nucleus of the cell **[1]**. While progressing into mid S phase, the spots become blurred and localize more towards the perimeter of the nucleus and the nucleoli **[2]**.

6.2.1. LAB MEDIA: Figure 1B. *Video Editor: Show either one of the two rows of panels. Emphasize the early S panel*

6.2.2. LAB MEDIA: Figure 1B. *Video Editor: Emphasize the mid S panel in the same row*

6.3. In late S phase, the spots reduce in numbers but become increasingly large as PCNA concentrates at late replication sites **[1]**.

6.3.1. LAB MEDIA: Figure 1B. *Video Editor: Emphasize the late S panel in the row selected in 6.2.1*

6.4. Low doses of energy, such as 1000 microseconds of dwell time **[1]**, do not induce recruitment of EGFP-FBXL10 *(E-G-F-P-F-B-X-L-ten)*, a double-stranded break responder **[2]**, but are sufficient to induce recruitment of NTHL1-mCherry *(N-T-H-L-one-M-cherry)*, a base excision repair pathway protein that is recruited to sites of oxidative DNA damage **[3]**.

6.4.1. LAB MEDIA: Figure 2A. *Video Editor: Emphasize the label 1000 µs dwell time*

6.4.2. LAB MEDIA: Figure 2A. *Video Editor: Emphasize the EGFP-FBXL10 row in the 1000 µs dwell time*

6.4.3. LAB MEDIA: Figure 2A. *Video Editor: Emphasize the NTHL1-mCherry row in the 1000 µs dwell time*

6.5. At 3000 microseconds dwell time **[1]**, both EGFP-FBXL10 **[2]** and NTHL1-mCherry are recruited, demonstrating a laser output that generates both oxidative lesions and double-stranded breaks **[3]**.

6.5.1. LAB MEDIA: Figure 2A. *Video Editor: Emphasize the label 3000 µs dwell time*

6.5.2. LAB MEDIA: Figure 2A. *Video Editor: Emphasize the EGFP-FBXL10 row in the 3000 µs dwell time*

6.5.3. LAB MEDIA: Figure 2A. *Video Editor: Emphasize the NTHL1-mCherry row in the 3000 µs dwell time*

6.6. EXO1b *(exo-one-B)* reaches a maximum level of accumulation at micro-irradiation sites around 1 minute **[1]** and then slowly starts disengaging from the DNA lesions **[2]**. In the presence of olaparib, accumulation of EXO1b at the laser stripe at 1 minute is significantly less **[3]** compared to the vehicle control **[4]**.

6.6.1. LAB MEDIA: Figure 4A. *Video Editor: Emphasize the 25s and 150s DMSO-EXO1b panels*

6.6.2. LAB MEDIA: Figure 4A. *Video Editor: Emphasize the 350s DMSO-EXO1b panel*

6.6.3. LAB MEDIA: Figure 4C. *Video Editor: Emphasize the pink curve at 60 sec*

6.6.4. LAB MEDIA: Figure 4C. *Video Editor: Emphasize the blue curve at 60 sec*

# Conclusion

**7. Conclusion Interview Statements**

7.1. **Bearach Miwatani-Minter:** It is crucial that the microscope is given ample time to heat up and that culturing conditions are optimal for each experiment to ensure consistent results. Additionally, it is important to optimize your laser settings with different DNA damage reporters to test for specific DNA lesions.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 3.1.1, 3.1.2*

7.2. **Bearach Miwatani-Minter:** Following micro-irradiation, consider validating results with classical biochemical methods such as fractionation, immunoprecipitation or ChIP. These approaches sample a larger cell population, thus providing statistical robustness.

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