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TITLE:

Fluorescence Recovery after Photobleaching of Yellow Fluorescent Protein Tagged p62 in Aggresome-like Induced Structures

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fluorescence recovery after photobleaching, FRAP, yellow fluorescent protein, live cell imaging, protein mobility, murine macrophages, p62, sequestosome-1, aggresome

SUMMARY:

We describe a comprehensive and practical protocol for fluorescence recovery after photobleaching experiments with live cells. Although the protocol was used to measure the mobility of yellow fluorescent protein-tagged p62 in aggresome-like induced structures, it can be applied to a variety of microscopy systems and fluorescent proteins.

ABSTRACT:

Fluorescence recovery after photobleaching (FRAP) is a microscopy technique that can be used to quantify protein mobility in live cells. In a typical FRAP experiment, steady-state fluorescence is observed by repeated imaging with low-intensity laser light. Subsequently, the fluorescent molecules are rapidly and irreversibly impaired via brief exposure to high-intensity laser light. Information about protein mobility is obtained by monitoring the recovery of fluorescence. We used FRAP to determine the mobility of p62 in aggresome-like induced structures (ALIS) in murine macrophages after stimulation with lipopolysaccharide (LPS). Because many existing FRAP protocols are either incomplete or complex, our goal was to provide a comprehensive, practical, and straightforward step-by-step protocol for FRAP experiments with live cells. Here, we describe RAW264.7 macrophage transfection with yellow fluorescent protein-p62 (YFP-p62), induction of ALIS by exposing the cells to LPS, and a step-by-step method for collecting prebleach and postbleach FRAP images and data analysis. Finally, we discuss important factors to consider when conducting a FRAP experiment.

INTRODUCTION:

Fluorescence recovery after photobleaching (FRAP) is a microscopy technique that can be used to quantify protein dynamics in live cells^{1,2}. The popularity of FRAP has increased because of the widespread commercial availability of laser scanning confocal microscopes with high resolution, speed, and sensitivity, and a “rainbow” of genetically encoded fluorescent proteins, such as green fluorescent protein (GFP) and yellow fluorescent protein (YFP)³. Genetically encoded fluorescent proteins are fused to a protein of interest to allow for the subcellular localization of the protein of interest. In a typical FRAP experiment, the steady-state fluorescence in an acquisition region of interest (ROI) within a cell is observed via repeated imaging of that ROI with low-intensity laser light. Subsequently, the fluorescent molecules are rapidly and irreversibly impaired in a predefined subset of the acquisition ROI, hereafter referred to as the bleach ROI, by brief exposure to high-intensity laser light. As new unbleached proteins replenish bleached proteins over time, the speed and intensity of fluorescence recovery in the bleach ROI provides information about protein mobility (**Figure 1**)⁴.

Our interest in determining the mobility of the ubiquitin binding protein p62 (also known as sequestosome-1) in aggresome-like induced structures (ALIS) in murine RAW264.7 macrophages after stimulation with lipopolysaccharide (LPS) led us to review the FRAP literature. Unfortunately, many of the existing FRAP protocols are incomplete or inordinately complex⁵⁻⁹. Some do not provide detailed information about the laser settings, beam path configuration, and image acquisition parameters⁵⁻⁹. Others omitted key details regarding data analysis, such as how to address the issue of bleach ROI drift^{6,9} or how to calculate important recovery parameters, including the mobile fraction (M_f), immobile fraction (I_f), and half-time of recovery ($t_{1/2}$)^{5,7}. Conversely, others placed too much emphasis on complex mathematical formulas used to calculate M_f , I_f , and $t_{1/2}$ ^{5,6,8,9}. Thus, our purpose is to provide a comprehensive, practical, and straightforward step-by-step protocol for FRAP experiments with live cells.

PROTOCOL:

1. RAW264.7 Macrophage Transfection

1.1. Culture 100,000 RAW264.7 cells (**Table of Materials**) in complete culture medium (Dulbecco's Modified Eagle Medium (DMEM)) (**Table of Materials**) containing 4.5 g/L glucose supplemented with 10% fetal bovine serum (**Table of Materials**) and penicillin/streptomycin (**Table of Materials**) onto untreated 35-mm glass-bottom dishes (**Table of Materials**) and place the dishes in a 37 °C/5% CO₂ incubator. On the following day, transfect the cells using 1 mg/mL polyethylenimine (PEI) (**Table of Materials**).

1.2. Mix 1.5 µg of YFP-p62 with 8 µL of PEI into 166 µL of serum-free DMEM (base medium without fetal bovine serum and penicillin/streptomycin).

1.3. Let the transfection complex mixture sit at room temperature for 15 min prior to adding the mixture into each plate.

1.4. Add the complexes to the existing medium with cells and rock the plate gently.

1.5. Place the plate in a 37 °C /5% CO₂ incubator overnight.

1.6. The following morning, aspirate the medium off the plate, then rinse once with complete medium. Add 2 mL of complete medium to the plate and allow the cells to recover until the next day.

2. Induction of ALIS with Lipopolysaccharide (LPS)

2.1. The next day, aspirate the medium from the plates, then add 1 mL of complete medium containing 10 ng/mL LPS (**Table of Materials**). Allow the plates to incubate for 5 h in a 37 °C/5% CO₂ incubator.

2.2. After the LPS treatment, aspirate the medium, then add 1 mL of cold, sterile Tyrode's buffer containing 145 mM NaCl, 5 mM KCl, 10 mM glucose, 1.5 mM CaCl₂, 1.0 mM MgCl₂, and 10 mM HEPES (pH 7.4) to rinse the plate.

2.3. Aspirate the buffer, then add 1 mL of cold, sterile Tyrode's buffer supplemented with 10 µg/mL nocodazole (**Table of Materials**). Allow the plates to incubate at 4 °C for 15-20 min prior to FRAP imaging and analysis.

NOTE: The use of Tyrode's buffer containing HEPES as the buffering compound allows for imaging to be performed room temperature. Nocodazole was used to decrease ALIS movement by microtubules. Culture medium contains bicarbonate as a buffering compound requiring CO₂ to buffer. Otherwise, the bicarbonate contained in medium changes pH in the absence of CO₂.

3. Set-up of the Confocal Microscope and Selecting the Region of Interest

3.1. Laser Selection and Beam Path Configuration

3.1.1. Use any suitable confocal microscope (**Table of Materials**) equipped with AIM or ZEN software (**Table of Materials**) or any suitable imaging software.

3.1.2. Select the 514-nm line of the Argon/2 laser, as YFP has peak excitation at 512 nm and peak emission at 527 nm. Click the **Acquire** button, then click the **Laser** button. In the Laser Control window, click **Argon/2 458, 477, 488, 514 nm**, then click the **Standby** button. After waiting ~3 min for the laser to warm up, click the **On** button (**Figure 2A**).

3.1.3. Set the laser power of the 514-nm Argon/2 laser line to 100% (8.6 A). Click the **Acquire** button, then click the **Laser** button. In the Laser Control window, enter **100** in the **Output [%]** field, then press the **Enter** button (**Figure 2A**).

3.1.4. Set the transmission of the 514-nm Argon/2 laser line to 5%. Click the **Acquire** button, then

the **Channels** button. In the **Scan Control** window, click the **Channels** button, then click the square white box to the left of the text **514 nm** in the **Line active** column to activate the 514-nm laser line. Enter 5 in the **Transmission [%]** field for the 514-nm laser line (**Figure 2D**).

3.1.5. Set the primary dichroic beam splitter (Haupt Farb Teiler (HFT)) to the **HFT 458/514/561** position such that these bands are deflected to the specimen for excitation. Click the **Acquire** button, then the **Config** button. In the **Configuration Control** window, click the **Channel Mode** button, then the **Single Track** button. Click the **HFT** button, then select **HFT 458/514/561** from the drop-down menu (**Figure 2B**).

3.1.6. Set the first secondary dichroic beam splitter (Neben Farb Teiler 1 (NFT1)) to the **mirror** position, which will deflect 100% of the light to the second secondary dichroic beam splitter (Neben Farb Teiler 2 (NFT2)). Click the **Acquire** button, then the **Config** button. In the **Configuration Control** window, click the **Channel Mode** button, then the **Single Track** button. Click the **NFT1** button, then select **Mirror** from the drop-down menu (**Figure 2B**).

3.1.7. Set the second secondary dichroic beam splitter (NFT2) to the **NFT 515** position to ensure that wavelengths < 515 nm will be reflected and wavelengths > 515 nm will be transmitted. Click the **Acquire** button, then the **Config** button. In the **Configuration Control** window, click the **Channel Mode** button, then the **Single Track** button. Click the **NFT2** button, then select **NFT 515** from the drop-down menu (**Figure 2B**).

3.1.8. Set the long pass (LP) emission filter (EF) to **LP 530**, so that wavelengths > 530 nm will be transmitted to the photomultiplier tube (PMT, detector). Click the **Acquire** button, then the **Config** button. In the **Configuration Control** window, click the **Channel Mode** button, then the **Single Track** button. Click the **Emission filter** button, then select **LP 530** from the drop-down menu (**Figure 2B**).

3.1.9. Select **Channel 3**. Click the **Acquire** button, then the **Config** button. In the **Configuration Control** window, click the **Channel Mode** button, then the **Single Track** button. Click on the square white box to the left of the **Ch3** button.

3.2. Image Acquisition Set-Up

3.2.1. Select the Plan-Apochromat 63×/1.40 oil objective (**Table of Materials**). View the specimen through the microscope eyepiece and place the ALIS in the center of the field of view. Click the **Acquire** button, then the **Micro** button. In the **Microscope Control** window, click on **Objective**, then select the Plan-Apochromat 63×/1.40 oil objective from the drop-down menu.

3.2.2. Set the frame size to **512 × 512 pixels**, the scan speed to **8**, and the data depth to **12 bit**. Click the **Acquire** button, then the **Scan** button. In the **Scan Control** window, click the **Mode** button and the **Frame** button, then click on the **512** button. Enter **8** in the **Scan Speed** field, press **Enter**, then click on the **12 Bit** button (**Figure 2C**).

3.2.3. Set the scan average to **1** and the optical zoom to **3**. Click the **Acquire** button, then click the **Scan** button. In the **Scan Control** window, click the down arrow button of the scan average number field, then select **1** from the drop-down menu. Enter **3** in the optical zoom field, then press **Enter** (**Figure 2C**).

3.2.4. Set the pinhole to **1.95 Airy units** and detector gain to **582** (just below saturation). Click the **Acquire** button, then the **Scan** button. In the **Scan Control** window, click the **Channels** button, enter **196** in the Pinhole field, then press **Enter**. Enter **582** in the Detector Gain field, then press **Enter** (**Figure 2D**).

3.2.5. Ensure that the 514-nm Argon/2 laser line is set to 100% power (8.6 A). Click the **Acquire** button, then the **Laser** button. In the **Laser Control** menu, the value in the Output [%] field should be **100** (**Figure 2A**).

3.2.6. Ensure that the 514-nm Argon/2 laser line is set to 5% transmission. Click the **Acquire** button, then the **Scan** button. In the **Scan Control** window, the value in the Transmission [%] field for the 514-nm laser line should be **5** (**Figure 2D**).

3.2.7. Create a square-shaped ROI (acquisition ROI) that has the dimensions 150 × 150 pixels (194.6 μm²). Click the **Acquire** button, then the **Edit ROI** button. In the **Edit ROI** menu, click the rectangle icon, then click and drag in the image window to create a square that has the dimensions 150 × 150 pixels (194.6 μm²) (**Figure 1A**).

NOTE: The acquisition ROI includes (a) an ALIS of interest, (b) an area of fluorescence that is not an ALIS (control ROI) and is at least 20 × 20 pixels (3.3 μm²), and (c) an area of little to no fluorescence (background ROI) that is at least 20 × 20 pixels (3.3 μm²) (**Figure 1A**). It is important to include a control ROI in the acquisition ROI so that the photobleaching that may occur with repeated imaging can be controlled. The acquisition ROI that is defined here will be the only area that will be scanned. Repeated laser scanning within an acquisition ROI only will restrict photobleaching to the ROI rather than, for example, photobleaching the entire cell or several cells, and the number of pixels collected at the PMT (detector) will be higher compared to scanning an entire cell or multiple cells.

3.2.8. Set-up the time series such that the acquisition ROI is scanned 35 times, once every 30 s. Click the **Acquire** button, then the **Time Series** button. In the **Time Series Control** window, click the **Manual start series** button and the **Manual stop series** button. Enter **35** in the manual stop series field, press the **Enter** key, then click the **30.0 sec** cycle delay button (**Figure 2E**).

3.2.9. Set the bleach control such that the photobleaching will occur after scan number 5, to collect 5 prebleach images of the acquisition ROI. Click the **Acquire** button, then the **Edit Bleach** button. In the **Bleach Control** window, click the white square next to the **Bleach after number scans**. Enter **5** in the scan number field, then press the **Enter** key (**Figure 2F**).

NOTE: Prebleach and postbleach images should be acquired at the same optical depth.

3.2.10. Create a circular-shaped bleach ROI within the ALIS that has a 10-pixel diameter (area = $0.8 \mu\text{m}^2$). Click the **Acquire | Edit Bleach | Define Regions**. In the **Bleach Regions** window, click the **Circle** icon. Click and drag in the image window to create a circle that has a 10-pixel diameter (area = $0.8 \mu\text{m}^2$) (**Figure 2F**).

3.2.11. Set iterations to **300**. Click the **Acquire** button, then the **Edit Bleach** button. In the **Bleach Control** window, enter **300** in the Iterations field, then press the **Enter** key (**Figure 2F**).

3.2.12. Set the Argon/2 514-nm laser line to 100% power (8.6 A) and 100% transmission during the bleach. Click the **Acquire** button, then the **Edit Bleach** button. In the **Bleach Control** window, click the white square box to the left of **514 nm**. Enter **100** in the Transmission [%] field for the 514-nm laser line, then press the **Enter** button (**Figure 2F**).

NOTE: The number of iterations needs to be determined empirically. It will vary depending on the fluorophore, the size of the structure that will be bleached, and the laser.

3.3. Data Collection

3.3.1. Start the FRAP experiment. Collect the first 5 prebleach images and the first postbleach image, then calculate bleach depth according to the following equation.

$$\text{Bleach depth} = 100 * \left(1 - \frac{\text{First Postbleach Value of the Bleach ROI}}{\text{Prebleach Average of the Bleach ROI}} \right).$$

If bleach depth is < 90 , stop the experiment and discard the data, as the bleach depth was not sufficient. When bleach depth is ≥ 90 , collect data for analysis with an image processing program and a spreadsheet program (**Table of Materials**).

NOTE: When drift of the ALIS is $\geq \sim 3 \mu\text{m}$, stop the experiment and discard the data, as this amount of image drift cannot be corrected for by analysis with image processing software (**see materials list**). When drift of the ALIS is $< \sim 3 \mu\text{m}$, collect the data for data analysis with an image processing program and a spreadsheet program (**Table of Materials**).

3.3.2. Collect FRAP data from 10 ALIS from 10 cells with less than $\sim 3 \mu\text{m}$ of drift of the ALIS. Next, transfer the AIM .lsm files to a personal computer for data analysis.

3.4. Data Analysis in an Image Processing Program

3.4.1. Correct for image drift by aligning or matching (i.e., registering) the stack of time-series images of the acquisition ROI. To do so, open each AIM .lsm file with an image processing program (**Table of Materials**), then select **Plugins | Registration | StackReg | Translation**. Next, select **Plugins | Registration | StackReg | Rigid Body**¹⁰.

3.4.2. Set-up ROI Manager to measure the signal intensity in the bleach ROI. Select **Analyze | Tools | ROI Manager**. Select the **Oval** tool, then draw a circle in the bleach ROI with a diameter

of 10 pixels, then click the **Add** button.

3.4.3. Set-up ROI Manager to measure signal intensity in the control ROI. In ROI Manager, select the **Rectangle** tool, then draw a square of 20×20 pixels in the control ROI region, then click the **Add** button.

3.4.4. Set-up ROI Manager to measure signal intensity in the background ROI. In ROI Manager, select the **Rectangle** tool, draw a square of 20×20 pixels in the background ROI region, then click the **Add** button.

3.4.5. Rename the ROIs. After adding the ROIs to be analyzed in **ROI Manager**, rename them **Bleach ROI**, **Control ROI**, and **Background ROI** accordingly.

3.4.6. Measure signal intensity in the ROIs. Select **Bleach ROI**, **Control ROI**, and **Background ROI**, then select **More | Multi Measure**. Ensure that **Measure all 35 slices** and **One row per slice** are selected. In the **Set Measurements** window, ensure that only **Mean gray value** is selected.

NOTE: These are the raw FRAP data (**Figure 1B**).

3.4.7. Paste the results into a spreadsheet program (**Table of Materials**). Copy the bleach ROI, control ROI, and background ROI signal intensity results then paste them to columns labeled **Bleach ROI**, **Control ROI**, and **Background ROI**, respectively in a spreadsheet program (**Table of Materials**).

3.5. Data Analysis in a Spreadsheet Program

3.5.1. Background-correct the signal intensity in the bleach ROI and control ROI (**Figure 1A, C**). Use the **Insert Function** tool in a spreadsheet program (**Table of Materials**) to subtract the values in the column labeled **Background ROI** from the values in the column labeled **Bleach ROI**. Subtract the values in the column labeled **Background ROI** from the values in the column labeled **Control ROI**. Label these new columns **Corrected Bleach ROI** and **Corrected Control ROI**.

3.5.2. Normalize the signal in the bleach ROI to the background-corrected signal in the control ROI (**Figure 1A, D**). Use the **Insert** function in a spreadsheet program (**Table of Materials**) to divide the values in the column labeled **Corrected Bleach ROI** by the values in the column labeled **Control Corrected ROI**. Label this new column **Normalized Corrected Bleach ROI**.

3.5.3. Normalize the signal in the **Normalized Corrected Bleach ROI** column to the average of the 5 prebleach values in the bleach ROI (**Figure 1A, E**). Calculate the average of the 5 prebleach values in the bleach ROI. Next, divide the values in the column labeled **Normalized Corrected Bleach ROI** by the average of the 5 prebleach values in the bleach ROI. Label this new column **Normalized Corrected Prebleach Average Bleach ROI**.

3.6. Mobile Fraction and Half Time of Recovery from Curve-Fitted Data

3.6.1. Curve-fit the normalized and corrected bleach ROI data using an image processing program (Table of Materials). In the imaging processing program (Table of Materials), select **Analyze | Tools | Curve Fitting**. Copy the postbleach normalized and corrected bleach ROI values and the corresponding time values, then paste them into the **Curve Fitter** window. Select **Exponential Recovery** from the **Curve Fitter** drop-down menu, then select **Fit**.

3.6.2. Calculate the M_f from the parameters of the recovery function, which provides values for: 'a,' a slowly recovering fraction; 'b,' the recovery rate; and 'c,' a rapidly diffusing fraction. Calculate M_f by plugging in the values for 'a' and 'c' into the equation $M_f = a + c$. Calculate the I_f using the equation $I_f = 1 - M_f$. Calculate $t_{1/2}$ by substituting the value for 'b' into the equation $t_{1/2} = \frac{\ln(0.5)}{-b}$ then solving for $t_{1/2}$.¹¹

REPRESENTATIVE RESULTS:

Shown here are the results of a typical experiment in which we used FRAP to examine the degree of mobility of p62 in ALIS in RAW264.7 cells treated with LPS for 3-5 h¹². **Figure 3A** shows the raw data obtained from a bleach, control, and background ROI after the stack of images had been aligned to correct for small amounts (< ~3 μ m) of image drift of the ALIS. YFP-p62 fluorescence in this ALIS at prebleach and postbleach is shown in **Figure 3E** and **Supplementary Video 1**. **Figure 3B** shows these data after background-correction. **Figure 3C** shows these data after correcting for fluorescence in the control ROI. **Figure 3D** shows these data normalized to the mean fluorescence of the 5 prebleach values in the bleach ROI. The degree of bleaching was sufficient in this experiment (bleach depth = 91.94). YFP-p62 fluorescence within this ALIS recovered slowly, as $t_{1/2} = 128.27$ s (2.14 min). YFP-p62 was not a very mobile protein in this experiment, as $M_f = 21.97$ and $I_f = 78.03$.

FIGURE AND TABLE LEGENDS:

Figure 1. A diagram of a RAW264.7 cell and steps for image analysis after the FRAP images have been aligned to correct for image drift of the ALIS. (A) Diagram of a RAW264.7 cell depicting several ALIS and the bleach, control, and background ROIs within the acquisition ROI. (B) An idealized depiction of raw FRAP data obtained in the bleach, control, and background ROIs in panel A. (C) An idealized graph of raw FRAP data that have been background-corrected. (D) An idealized graph of background-corrected FRAP data that have been normalized to fluorescence in the control ROI. (E) An idealized graph of normalized and background-corrected FRAP data that have been normalized to the prebleach fluorescence in the bleach ROI. This figure was modified from Rabut and Ellenberg⁴.

Figure 2. Images of the user interface in the AIM software (Table of Materials) for laser selection, beam path configuration, and image acquisition parameters for the FRAP experiments. (A) Laser control screen showing the Argon/2 laser lines, output, and tube current used. (B) Configuration control screen showing the beam splitter and emission filter settings used. (C) Scan control screen showing the setting used for image acquisition. (D) Scan control screen showing the pinhole, detector gain, amplifier offset, amplifier gain, and transmission

settings for the Argon/2 514-nm laser line. (E) Time series control screen showing the time series settings used. (F) Bleach control screen showing the prebleach and postbleach parameters used. Abbreviations: HFT, Haupt Farb Teiler (primary dichroic beam splitter); NT1, Neben Farb Teiler 1 (first secondary dichroic beam splitter); NT2, Neben Farb Teiler 2 (second secondary dichroic beam splitter); EF, emission filter.

Figure 3. A representative FRAP experiment to study YFP-p62 dynamics in ALIS in RAW264.7 cells. (A) Raw fluorescence intensity data for a bleach, control, and background ROI. (B) The data given in panel A after the bleach and control ROI had been corrected for fluorescence in the background ROI. (C) The data given in panels A and B after normalizing the fluorescence intensity in the bleach ROI to account for fluorescence in the background-corrected control ROI. (D) The data given in panels A-C after normalizing the fluorescence intensity in the normalized and background-corrected bleach ROI to the average of the first 5 prebleach values in the bleach ROI. $M_f = 21.97$, $I_f = 78.03$, $t_{1/2} = 128.27$ s (2.14 min), and bleach depth = 92.94. (E) An image of an ALIS at prebleach (1.5 min) and postbleach (0, 6, and 16 min). The scale bar equals 5 μ m and is valid for all panels. Abbreviations: Con, control; BG, background; Corr., corrected; Norm., normalized; Avg., average. This figure was modified from Cabe *et al.*¹²

Supplementary Video 1: A typical ALIS in a RAW264.7 macrophage before and after photobleaching. YFP-p62 fluorescence is slow to recover after the photobleach; hence, p62 is not a very mobile protein. For this experiment, $M_f = 25.62$, $I_f = 74.38$, $t_{1/2} = 442.79$ s (7.38 min), and bleach depth = 90.19. The scale bar = 5 μ m. This video was modified from Cabe *et al.*¹²

DISCUSSION:

We provide a comprehensive, practical, and simple step-by-step protocol for FRAP experiments with live cells. Herein, the protocol was used to measure the mobility of YFP-p62 in ALIS in RAW264.7 macrophages, but it can be applied to many of the laser scanning confocal microscope systems and genetically encoded fluorescent proteins that are now available. For any microscopy system, pilot experiments are critical for determining the optimal FRAP parameters, including acquisition, bleach, and control ROI sizes, laser intensity for photobleaching, and prebleach and postbleach image acquisition. It is reasonable to expect the optimal FRAP parameters to differ for each genetically encoded fluorescent protein and cell line.

Important factors to consider when conducting a FRAP experiment include (a) achieving suitable bleach depth, (b) the use of a brief bleaching step, (c) allowing sufficient time postbleach to observe the full recovery function, (d) photobleaching efficiency, (e) cytotoxicity with repeated FRAP, and (f) the inclusion of a control for fluorescence loss due to repeated imaging. We recommend that bleach depth, which can be calculated according to the equation provided in section 3.3.1, be ≥ 90 ¹³. When bleach depth is < 90 , the degree of postbleach fluorescence recovery will be underestimated, and the values of I_f , M_f , and $t_{1/2}$ will be incorrect. Although the duration and intensity of the bleach-inducing laser pulses may vary between FRAP experiments, it is important that the photobleaching step be brief and substantially faster than the fluorescence recovery function. If it is not, then a significant amount of fluorescence recovery could occur during the bleaching step. With a long bleach time, fluorescence recovery during the

bleaching step would not be measured, and it would lead to incorrect measurements of I_f , M_f , and $t_{1/2}$. In addition, to obtain correct values for I_f , M_f , and $t_{1/2}$, the acquisition ROI should be observed postbleach until the fluorescence level in the bleach ROI has reached a plateau. For example, in our FRAP experiments, there was no difference between the I_f , M_f , and $t_{1/2}$ values when we observed YFP-p62 fluorescence in the bleach ROI for 32.2 min postbleach versus when we observed YFP-p62 in the bleach ROI for 15.1 min postbleach; thus, we concluded that the recovery function reached a plateau at 15.1 min postbleach¹². With regard to photobleaching efficiency, photobleaching increases with the square of the optical zoom factor¹⁴. Thus, the use of high optical zoom lenses is favorable for rapid photobleaching but can result in undesirable photobleaching during acquisition; the latter can be accounted for by imaging a control ROI. Repeated photobleaching is to be avoided as it can lead to the generation of cytotoxic reactive oxygen species (ROS). However, the degree of ROS generation due to exposure to a high-intensity laser is lower for genetically encoded fluorescent proteins than for chemical fluorophores (e.g., fluorescent antibodies)¹⁵, and the ROS generated are more likely to react within the genetically encoded fluorescent protein than with other molecules in the cell⁴. In addition to the increased probability of generating cytotoxic ROS, repeated photobleaching is to be avoided as it is difficult to control. Finally, although low laser transmission is used to acquire all non-bleach images, some photobleaching will invariably occur, which must be controlled. Possible controls for this include monitoring fluorescence in a control ROI within the acquisition ROI, obtaining control images in a neighboring unbleached cell, and performing control experiments with the identical settings for those used in the photobleaching experiments but without the photobleaching event.

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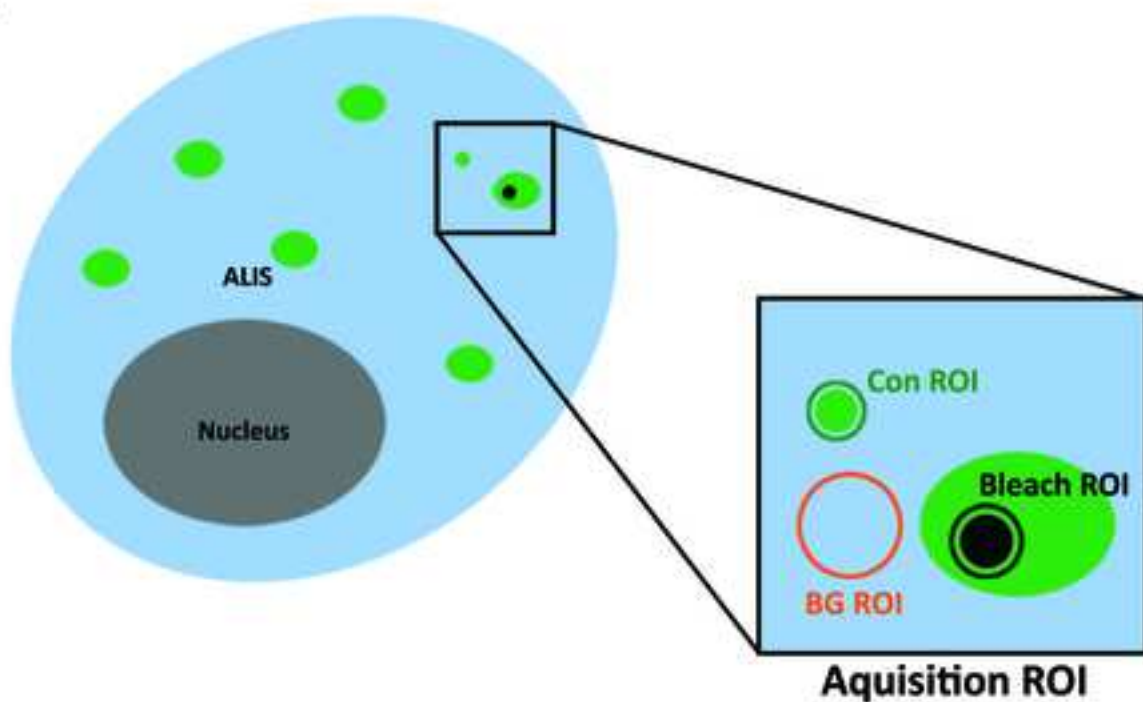
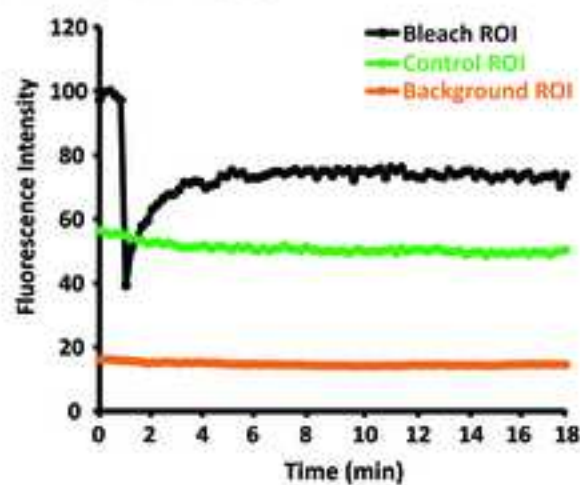
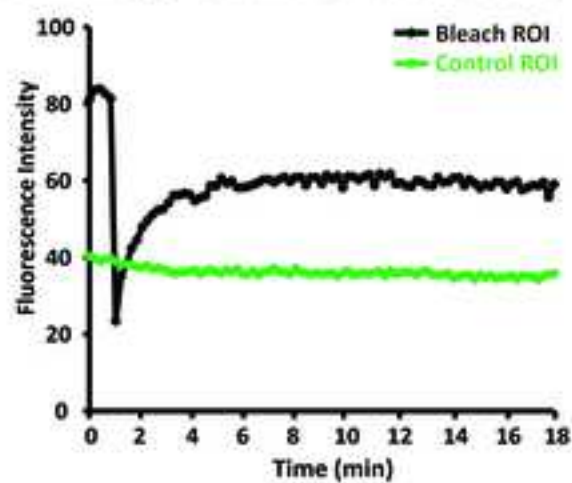
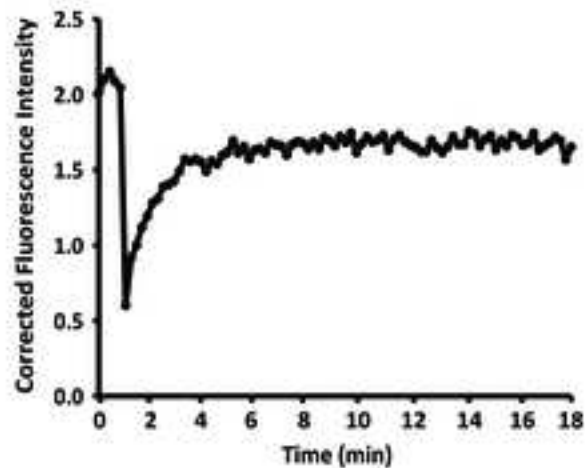
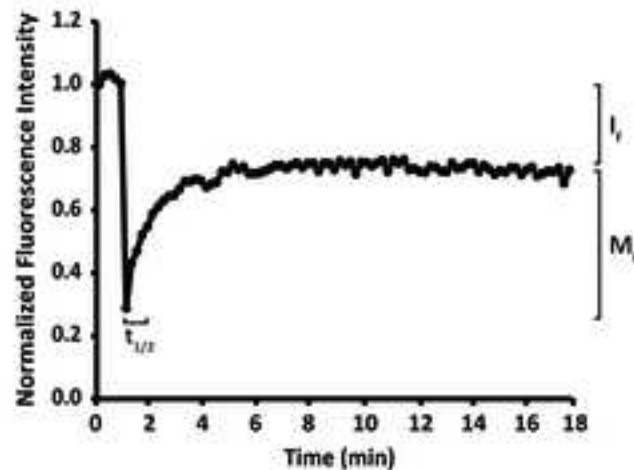
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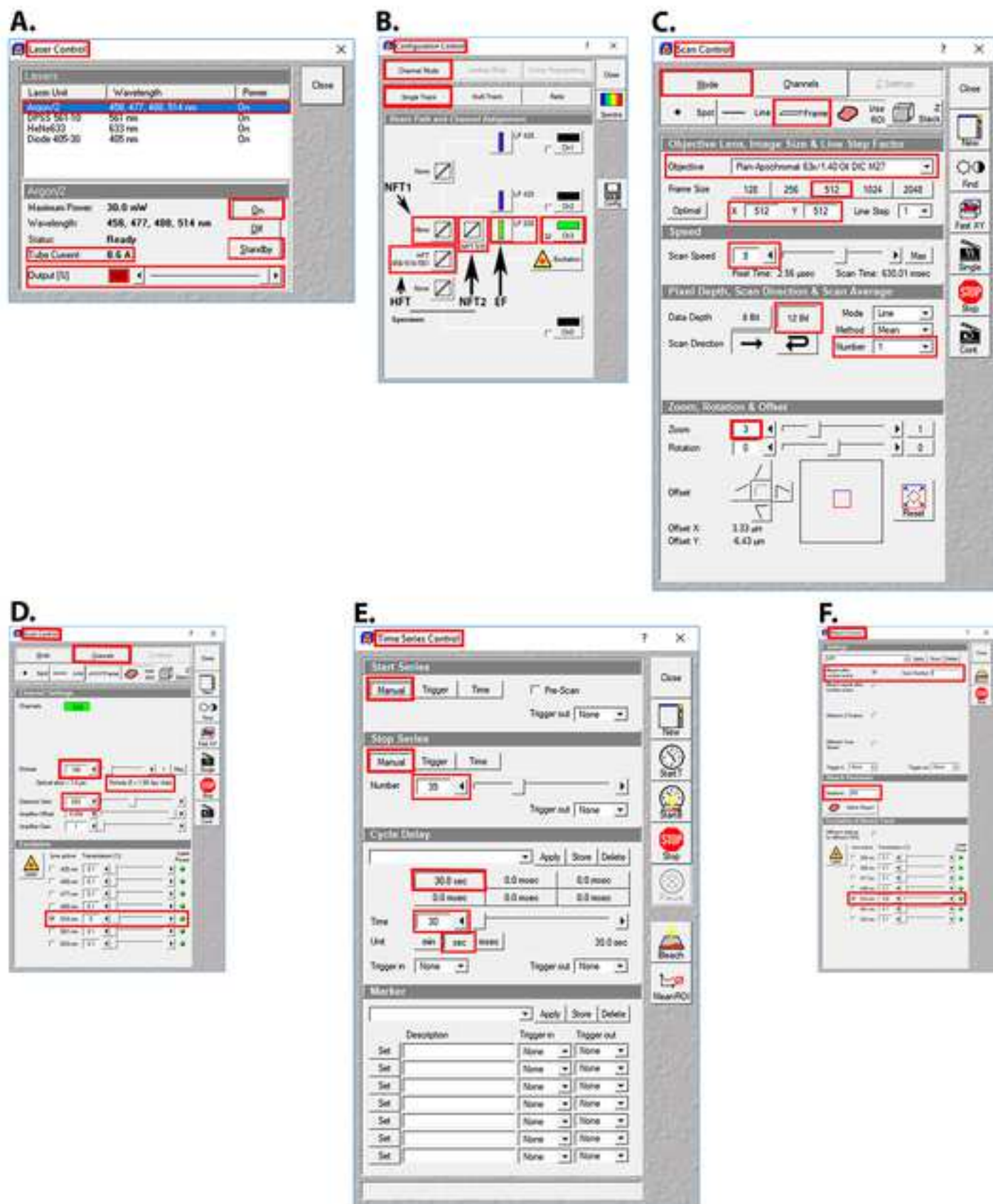
The authors have no conflict of interest to disclose.

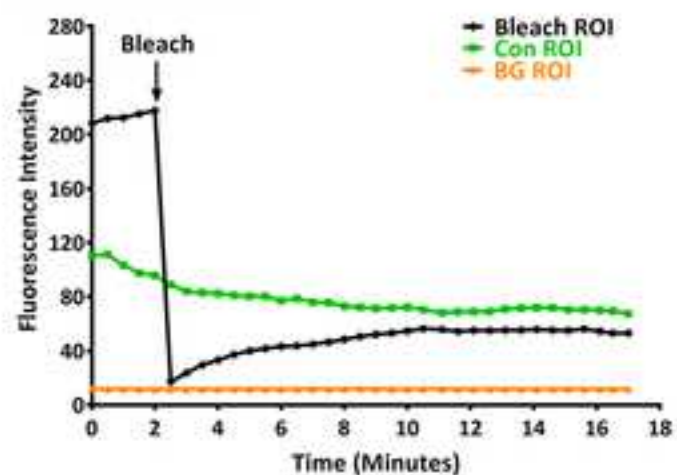
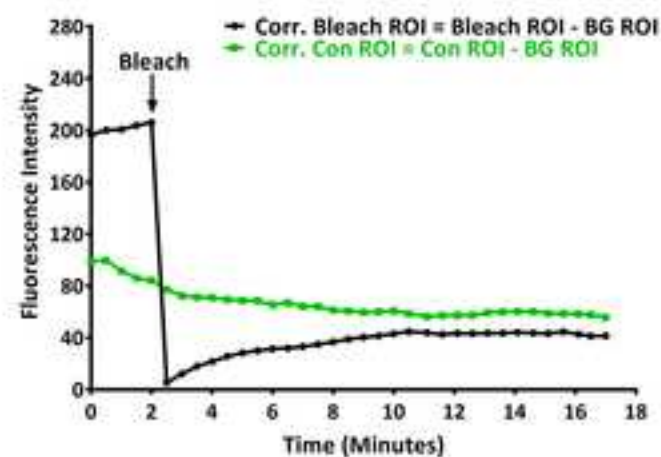
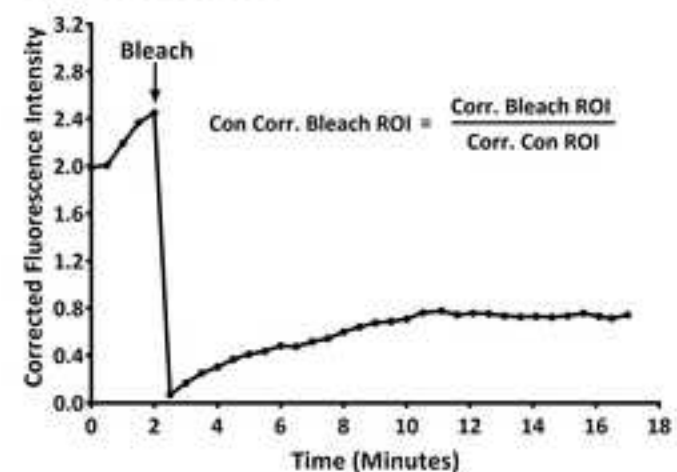
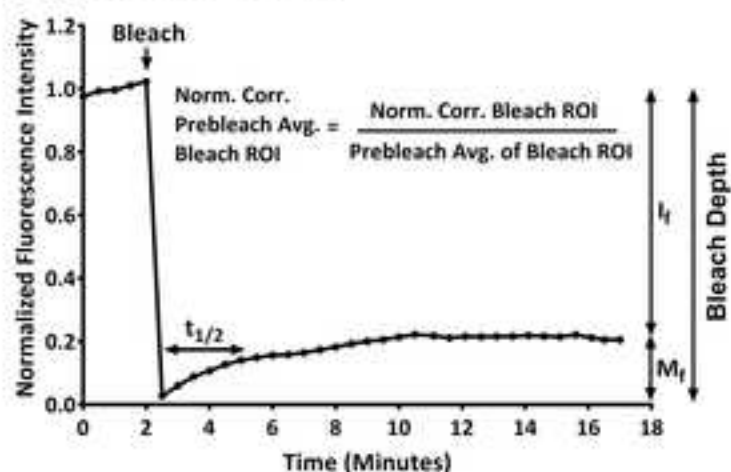
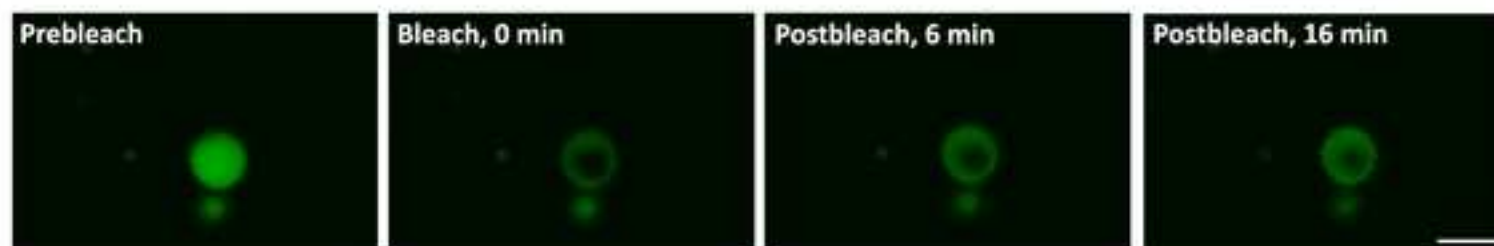
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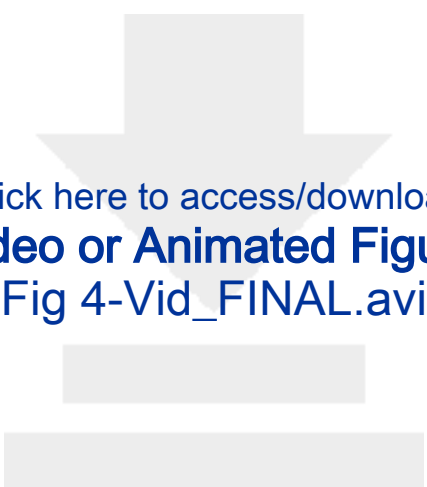
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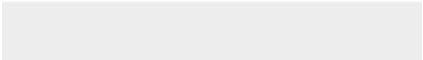

A.**B. Raw Data****C. Background Subtraction****D. Correction****E. Normalization**



A. Raw Data**B. Background Subtraction****C. Correction****D. Normalization****E.**



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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Dulbecco's Modified Eagle Medium	Hyclone	SH30022.01	High Glucose (4.5 g/L)
Fetal bovine serum	Gemini Bio-Products	100-106	
Glass bottom dishes	MatTek	P35G-1.5-14-C	35-mm
Image J software	National Institutes of Health	N.A.	
Lipopolysaccharide (LPS)	Sigma	L4391	
Nocodazole	Sigma	M1404	
Penicillin-streptomycin solution	Corning	30-002-CI	100×
Plan-Apochromat 63×/1.40 oil objective	Carl Zeiss MicroImaging, Inc	4407629904000000	
Polyethylenimine (PEI)	Polysciences	23966-1	linear (MW 25,000)
RAW 264.7 murine macrophages	ATCC	TIB-71	
Zeiss confocal microscope	Carl Zeiss MicroImaging, Inc	LSM 510	



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Author(s):

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Dear Dr. Dsouza,

Please find the enclosed revised version of the manuscript entitled “**Fluorescence Recovery After Photobleaching of Yellow Fluorescent Protein Tagged p62 in Aggresome-Like Induced Structures**” (JoVE59288 - [EMID: e197ad632e2721c9]) intended for publication in *Journal of Visualized Experiments*.

We have made the appropriate changes to the manuscript as per the suggestions made in the editorial and reviewer comments. Additionally, we have responded to all comments and technical points raised by the reviewers.

REPLIES TO EDITORIAL COMMENTS:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Authors' Reply: The manuscript has been proofread carefully to ensure that there are no spelling or grammar issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Authors' Reply: We have obtained proper documentation allowing for copyright permission. The manuscript has been modified to indicate an adaptation of figures from previous publications.

3. Please revise the title to be more concise.

Authors' Reply: The title has been modified and is as follows: “*Fluorescence Recovery After Photobleaching of Yellow Fluorescent Protein Tagged p62 in Aggresome-Like Induced Structures*”

4. Introduction: Please include applicable references to previous studies when describing existing FRAP protocols.

Authors' Reply: The appropriate references have been added to the manuscript. These references are listed as items 5-9 in the references section of the manuscript (page 11; line 444). The in-text citations have been updated accordingly throughout the manuscript.

5. Please define all abbreviations before use.

Authors' Reply: All abbreviations have been defined before use.

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Examples of commercial sounding language in your manuscript are: MatTek, Polysciences, Hyclone, Sigma, Carl Zeiss MicroImaging, Microsoft Corporation, Excel, etc.

Authors' Reply: All instances of commercial language have been removed from the manuscript.

7. 1.1: What culture medium is used? What are the conditions? Please specify.

Authors' Reply: The type of culture medium as well as required supplements (otherwise known as “complete medium”) and culturing conditions have been defined on page 2; line 78; step 1.1 of the protocol in the manuscript.

8. 1.6: What is complete medium? Serum-free DMEM?

Authors' Reply: “Complete medium” has been defined in page 2; line 78; step 1.1 of the protocol in the manuscript. “Serum-free DMEM” is defined as the base-medium without fetal bovine serum or antibiotics on page 2; line 85, step 1.2.

9. 3.1.2-3.1.5, 3.2.2-3.2.6: 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.).

Authors' Reply: All software steps have been explicitly explained and more specific details have been provided.

10. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Authors' Reply: The Protocol has been revised such that individual steps contain 2-3 actions per step and a maximum of 4 sentences per step.

11. After you have made all the recommended changes to your protocol (listed above), 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.

Authors' Reply: The authors have highlighted 2.75 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video.

12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Authors' Reply: The authors have highlighted complete sentences. The highlighted part of the step includes at least one action written in imperative sense.

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in

steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Authors' Reply: All relevant details required to perform the highlighted step have been included.

14. Figure 1: Please change the time unit “Min” to “min”.

Authors' Reply: This has been corrected in the revised manuscript.

15. References: Please do not abbreviate journal titles.

Authors' Reply: This has been corrected in the revised manuscript.

16. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Authors' Reply: This has been corrected in the revised manuscript.

REPLIES TO REVIEWERS' COMMENTS:

Reviewer #1:

Manuscript Summary:

The manuscript by Cabe et al. describes the procedure for conducting FRAP experiments using live cells. In this manuscript, they have described the procedures for the preparation of cells for analysis, expression of the fluorescently tagged protein p62, setting up of the confocal microscope, and analysis of data. Overall, this protocol is well-written. However, there are a few points that need to be clarified in order to describe a more general protocol for conducting FRAP experiments.

Minor Concerns:

Line 101: The authors should specify the reason why the culture medium was changed to cold Tyrode's buffer containing nocodazole. Would this culture medium be required for FRAP experiments that involve the use of other fluorescently tagged proteins as well?

Authors' Reply: The use of Tyrode's buffer containing HEPES as the buffering compound allowed us to perform imaging at room temperature and without the requirement of a CO₂. The regular culture medium contains bicarbonate as the buffering compound, which requires CO₂ to buffer. Performing live cell imaging in culture medium containing bicarbonate while also in the absence of CO₂ will change the overall pH. The manuscript has been modified to provide this rationale for using Tyrode's buffer containing nocodazole in page 3; line 113; after step 2.3.

Line 133: The authors should specify the temperature at which the FRAP experiment was conducted. Moreover, is a CO₂ chamber required to maintain incubation conditions during live cell imaging?

Authors' Reply: The manuscript has been modified to reflect the fact that the FRAP experiments were conducted at room temperature. A CO₂ chamber for short-term (lasting for ~60 min) live cell experiments such as those reported here but is necessary for longer term (lasting > 60 min) live cell experiments.

Reviewer #2:

Major Concerns:

1. In the representative FRAP images and movie (Figs. 3E and 4, respectively), fluorescence decreased in cell cytosol surrounding the bleach ROI. What is the reason behind this non-specific photobleach?

Authors' Reply: The decrease in fluorescence in the cell cytosol surrounding the bleach ROI is due to repeated imaging of the acquisition ROI, which was scanned 35 times, once every 30 seconds.

2. The authors set the pinhole at 1.95 airy units. We calculated that a pinhole of 1.95 airy units for a 63x/1.4NA objective is 27.5 μm (Airy unit= resolution limit x magnification). Did the thickness of the optical slice lead to loss of image acuity?

Authors' Reply: Please recall that we used a 63x/1.4NA objective with the optical zoom set to 3 and a pinhole set to 1.95 Airy units (Fig. 2C, D). These settings yield an optical slice of less than 1.4 μm , which provides a level of image acuity sufficient for FRAP experiments with ALIS.

3. The authors do not mention whether pre and post-bleach images were taken at different optical depths.

Authors' Reply: This omission has been corrected in the revised manuscript. Pre- and postbleach images were taken at the same optical depth.

4. The authors report that data were excluded in case of image drift > 3 μm (line 254). However, the representative video (Fig. 4) shows a drift of more than half the cell length- equal to 4-5 μm .

Authors' Reply: With regard to the topic of image drift, the experiment was stopped and the data were discarded when drift of the ALIS (not the bleach ROI) exceeded ~3 μm . We apologize for this error of phrasing. Based on a close examination of the representative video (Fig. 4), we estimate ALIS drift to be less than 1 μm ; the fluorescence recovery in the bleach ROI is non-uniform.

5. Line 335 states that the calculated bleach depth from raw fluorescence data presented in Figure. 3. However, we calculated the bleach depth to be 72 (assuming pre and post-bleach fluorescence to be 86 and 24, respectively).

Authors' Reply: Based on the reviewer's comment, we recalculated the bleach depth for the data presented in Figure 3. The prebleach average of the bleach ROI is equal to 213.08 and the first postbleach value of the bleach ROI is equal to 17.175. Thus, the bleach depth is 91.94 rather than 92.94, the value reported on page 7; line 338 of the manuscript. The manuscript has been corrected accordingly.

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By Carol C. Brown

Date: 11/29/18

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