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Project Page Link: <https://review.jove.com/account/file-uploader?src=21501633>

Title: Visualization and Quantitative Analysis of Genotoxin-induced PARP1/PARP2 Activation in Cells using a Fluorescent Fusion Protein-based Reporter

Authors and Affiliations:

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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, all done**
- 3. Filming location:** Will the filming need to take place in multiple locations? **Yes**
10 – 20 meters; same floor just different rooms.
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 18

Number of Shots: 40

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Robert Sobol:** Our lab studies PARP1 and PARP2-dependent DNA repair and DNA damage response pathways in normal and transformed cells.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.2.2*

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Robert Sobol:** A challenge in the field is the quantitative analysis of PARP1/PARP2 activation in cells while avoiding cell lysis. This allows for subcellular localization studies.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Robert Sobol:** This protocol allows us to quantify activation of the PARP1 and PARP2 signaling axis in response to genotoxins and following genetic alterations.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.11.3*

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Robert Sobol:** We have employed traditional immunofluorescence techniques with this assay to validate colocalization of proteins with poly(ADP-ribose).
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Robert Sobol:** Moving forward, we are using this assay for (i) genotoxicity testing, (ii) high-throughput analysis of PARP1/PARP2 or PARG inhibitors, and (iii) identifying novel factors involved in PARP1/PARP2 signaling.
 - 1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Lentivirus Production and Transduction to Express a PAR Reporter

Demonstrator: Wynand Roos

2.1. To begin, add 375 microliters of fetal bovine serum- and penicillin-streptomycin-free DMEM to a 1.5-milliliter sterile microcentrifugation tube [1]. Then, add 10 microliters of a lipid-based transfection reagent and all the required plasmids to the tube [2]. Tap the microcentrifugation tube gently to mix the medium, transfection reagent, and plasmid DNA [3]. Incubate at room temperature for 15 to 30 minutes to allow DNA and transfection reagent complex formation [4].

2.1.1. WIDE: Talent adding DMEM to the centrifugation tube.

2.1.2. Talent adding the lipid-based transfection reagent and plasmids to the microcentrifugation tube.

AND

TEXT ON A PLAIN BACKGROUND

pLV-EF1A-LivePAR-Hygro plasmid: 4 µg

pMD2.g(VSVG) plasmid: 2 µg

pRSV-REV plasmid: 2 µg

pMDLg/pRRE: 2 µg

Video Editor: Please play both shots side by side in a split screen

2.1.3. Talent gently tapping the microcentrifugation tube to mix the contents.

NOTE: Shots 2.1.3 and 2.1.4 were combined.

2.1.4. Talent placing the microcentrifugation tube aside for incubation at room temperature.

2.2. Next, add the plasmid and transfection reagent mix dropwise to the 60-millimeter dish containing the cultured 293FT (*Two-Nine-Three-F-T*) cells without removing the medium [1] and return the cells to the incubator for 48 hours [2].

2.2.1. Talent adding the plasmid and transfection reagent mix dropwise into the 60 millimeter dish containing 293FT cells.

2.2.2. Talent placing the dish in the incubator.

2.3. Then, collect the culture medium from the transfected 293FT cells to isolate the lentivirus particles [1]. Filter the collected medium through a sterile 0.45-micrometer

filter to separate cell debris from the lentiviral particles [2].

2.3.1. Talent collecting the culture medium from the transfected 293FT cells. **NOTE: Shots 2.3.1 and 2.3.2 were combined.**

2.3.2. Talent filtering the collected medium through a sterile 0.45 micrometer filter.

2.4. For transduction, obtain the desired cells cultured for 24 hours [1] and verify that the cell confluency is between 20% and 40% [2]. Next, add 1 milliliter of virus, 1 milliliter of growth medium, and 2 microliters of polybrene to a 15-milliliter conical tube [3]. Mix gently by inversion [4].

2.4.1. Talent removes the cells from the incubator.

2.4.2. Talent checking that the cells are between 20% and 40% confluent.

2.4.3. Talent adding virus, growth medium, and polybrene to a 15 milliliter conical tube.

2.4.4. Talent gently inverting the conical tube to mix the contents.

2.5. Now, remove the growth medium from the 6-well plate [1]. Add the virus, medium, and polybrene mix to each well [2]. Place the cells with the transduction mix in an incubator containing a humidified atmosphere at 32 degrees Celsius and 5% carbon dioxide for 16 to 18 hours [3].

2.5.1. Talent removing the growth medium from the 6-well plate.

2.5.2. Talent adding the virus, medium, and polybrene mix to each well of the plate.

2.5.3. Talent placing the 6-well plate in the incubator.

3. Preparing the Cells for Confocal Microscopy

Demonstrator: Wynand Roos

3.1. To validate the expression of the RNF146(100–182)-EGFP (*R-N-F-1-4-6 amino acids 100 to 182*), seed 200,000 LivePAR (*Live-Par*)- expressing cells per culture dish containing coverslips [1]. Allow the cells 24 to 36 hours to adhere to the coverslips, condition the medium, and begin replicating [2].

3.1.1. WIDE: Talent seeding LivePAR-expressing cells into a cell culture dish.

3.1.2. Talent placing the dish aside for incubation.

3.2. Then, expose the LivePAR-expressing cells to the given reagents [1]. Add the compounds directly to the medium containing the cells on the coverslips [2] and gently swirl the medium in the cell culture dishes to distribute the compounds [3]. Incubate the 60-millimeter dishes at 37 degrees Celsius for 60 to 90 minutes [4].

3.2.1. TEXT ON A PLAIN BACKGROUND
100 μ M NRH

10 μ M PARGi

10 μ M MNNG

For PARP1/PARP2-dependent PAR formation: Add 10 μ M PARPi

- 3.2.2. Talent adding the specified compounds to the cell culture dish containing LivePAR-expressing cells.
- 3.2.3. Talent gently swirling the cell culture dish to distribute the compounds.
- 3.2.4. Talent placing the dish in the incubator.
- 3.3. To fix the cells, remove the medium and wash the cells with PBS [1]. Prefix the cells in 4% formaldehyde in PBS for 15 minutes at room temperature [2]. Wash the cells three times with PBS [3].
 - 3.3.1. Talent aspirating the medium from the cell culture dish and adding PBS to wash the cells.
 - 3.3.2. Talent adding 4% formaldehyde in phosphate buffered saline to the cell culture dish.
 - 3.3.3. Talent washing the cells with phosphate buffered saline.
- 3.4. Then, add 3 milliliters of cold methanol and acetone to the cells in a 7 to 3 ratio to fix them [1]. Place the dishes at minus 20 degrees Celsius for 9 minutes [2].
 - 3.4.1. Talent adding cold methanol acetone solution to the cell culture dish.
 - 3.4.2. Talent placing the dish at minus 20 degrees Celsius.
- 3.5. Then, remove the methanol-acetone solution [1].
 - 3.5.1. Talent aspirating the methanol acetone solution from the dish.
- 3.6. After washing the cells three times with PBS, pipette 15 microliters of anti-fade medium containing DAPI onto the glass slide [1]. Gently mount the coverslip onto the mounting medium with the cells facing inward while minimizing bubbles [2].
 - 3.6.1. Talent pipetting anti-fade medium onto a glass slide.
 - 3.6.2. Talent placing the coverslip onto the mounting medium with cells facing inward.
- 3.7. Center and secure the coverslip onto the glass slide and seal the sides by applying a small amount of clear topcoat nail enamel to the edges [1]. Place the slides in the dark to allow the nail enamel to dry [2].
 - 3.7.1. Talent applying clear topcoat nail enamel to seal the edges of the coverslip.
 - 3.7.2. Talent placing the prepared slide in a dark storage area for drying.

4. Analysis of PAR Enriched Foci

Demonstrator: Wynand Roos

- 4.1. After downloading and installing ImageJ, open ImageJ and install the macro text file LivePAR_Macro (*Live-Par-Macro*) by clicking on **Plugins**, selecting **Macros**, and choosing **Install [1]**.
 - 4.1.1. SCREEN: 70497_Sobol.mp4: 00:33-00:45.
- 4.2. Open all confocal files in ImageJ **[1]** and save the files as .tif (*tif*) files to preserve the original files **[2]**.
 - 4.2.1. SCREEN: 70497_Sobol.mp4: 00:58-01:05
 - 4.2.2. SCREEN: 70497_Sobol.mp4: 01:14-01:20
- 4.3. Then, open a spreadsheet document and save it as date underscore cell line underscore foci analysis **[1]**.
 - 4.3.1. SCREEN: 70497_Sobol.mp4: 02:08-02:20, 02:45-02:50.
- 4.4. Analyze one .tif file at a time. Press **1** to find the nuclei. When the **ROI Manager** window opens, select all entries and press add to overlay the nuclei area over the original image **[1]**. Delete incorrectly identified nuclei **[2]**. Then, draw the nucleus using the freehand selection tool and press 2 to quantify poly ADP-ribose foci, excluding nuclei not fully within the frame. Select each nucleus in the ROI manager and count the number of foci per nucleus **[3]**.
 - 4.4.1. SCREEN: 70497_Sobol.mp4: 03:33-03:35, 03:45-03:54.
 - 4.4.2. SCREEN: 70497_Sobol.mp4: 04:08-04:10
 - 4.4.3. SCREEN: 70497_Sobol.mp4: 05:01-05:04, 05:12-05:20
- 4.5. Copy and paste the quantified data into the open spreadsheet document after scoring all nuclei in the file. Similarly, repeat the analysis for all .tif files **[1-TXT]**.
 - 4.5.1. SCREEN: 70497_Sobol.mp4: 14:45-15:10.
- 4.6. Plot poly ADP-ribose foci per nucleus in the software of choice **[1]**.
 - 4.6.1. SCREEN: 70497_Sobol.mp4: 16:53-17:06

Results

5. Results

- 5.1. Vehicle control cells showed pan-cellular EGFP (*E-G-F-P*) staining [1]. Genotoxin-treated cells showed accumulation of nuclear foci [2], representing localization within the nucleus [3].
 - 5.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the top row labeled control and show the green signal spread throughout the entire cells*
 - 5.1.2. LAB MEDIA: Figure 2. *Video editor: Highlight the bottom row showing treated cells with bright green spots*
 - 5.1.3. LAB MEDIA: Figure 2. *Video editor: Zoom in on the nuclei where the green dots are clustered*
- 5.2. Pre-treatment with the PARP1 (*Parp-One*) and PARP2 (*Parp-Two*) inhibitor Veliparib resulted in the loss of PAR foci [1]. Quantification of the number of PAR foci per nucleus as a function of time and treatment conditions showed similar results [2].
 - 5.2.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the bottom-most panels where the green nuclear spots are absent or reduced*
 - 5.2.2. LAB MEDIA: Figure 3B. *Video editor: Show the scatter plot displaying PAR foci per nucleus across the different time points on the horizontal axis*

1. Microliters

Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>

IPA: /'maɪ.kroʊ.liː.tər/

Phonetic Spelling: my·kroh·lee·ter

2. Microcentrifugation

Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>

- IPA: /ˌmaɪ.kroʊˌsɛn.trɪˈfjuːˈɡeɪ.ʃən/
Phonetic Spelling: my·kroh·sen·trih·fyoo·gay·shuhn
3. Transfection
Pronunciation link: <https://www.merriam-webster.com/dictionary/transfection>
IPA: /trænˈsfek.ʃən/
Phonetic Spelling: trans·fek·shuhn
 4. Plasmids
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /ˈplæz.mɪd/
Phonetic Spelling: ɜləz·mɪd
 5. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /ˈɪn.kjuː.bert/
Phonetic Spelling: in·kyoo·bayt
 6. Lentivirus
Pronunciation link: <https://www.merriam-webster.com/dictionary/lentivirus>
IPA: /ˈlen.təˌvaɪ.rəs/
Phonetic Spelling: len·tuh·vy·ruhs
 7. Lentiviral
Pronunciation link: <https://www.merriam-webster.com/dictionary/lentiviral>
IPA: /ˌlen.təˌvaɪ.rəl/
Phonetic Spelling: len·tuh·vy·ruhl
 8. Transduction
Pronunciation link: <https://www.merriam-webster.com/dictionary/transduction>
IPA: /trænzˈdʌk.ʃən/
Phonetic Spelling: tranz·duhk·shuhn
 9. Confluency
Pronunciation link: No confirmed link found
IPA: /kənˈfluː.ən.si/
Phonetic Spelling: kuhn·floo·uhn·see
 10. Polybrene
Pronunciation link: No confirmed link found
IPA: /ˈpɑː.liˌbriːn/
Phonetic Spelling: paa·lee·breen
 11. Confocal
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kənˈfoʊ.kəl/
Phonetic Spelling: kuhn·foh·kuhl
 12. Microscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/microscopy>
IPA: /maɪˈkraː.skə.pi/
Phonetic Spelling: my·krah·skuh·pee
 13. Formaldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/formaldehyde>

IPA: /fɔːrˈmæl.dəˌhɑːd/

Phonetic Spelling: for·mal·duh·hyde

14. Acetone

Pronunciation link: <https://www.merriam-webster.com/dictionary/acetone>

IPA: /ˈæs.əˌtoʊn/

Phonetic Spelling: as·uh·tohn

15. Phosphate

Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>

IPA: /ˈfɑːs.feɪt/

Phonetic Spelling: fahs·fayt

16. Methanol

Pronunciation link: <https://www.merriam-webster.com/dictionary/methanol>

IPA: /ˈmeθ.əˌnɔːl/

Phonetic Spelling: meth·uh·nawl

17. Anti-fade

Pronunciation link: No confirmed link found

IPA: /ˈæn.ti feɪd/

Phonetic Spelling: an·tee·fayd

18. DAPI

Pronunciation link: No confirmed link found

IPA: /ˈdæ.pi/

Phonetic Spelling: da·pee

19. Nucleus

Pronunciation link: <https://www.merriam-webster.com/dictionary/nucleus>

IPA: /ˈnuː.kli.əs/

Phonetic Spelling: noo·klee·uhs

20. Genotoxin

Pronunciation link: <https://www.merriam-webster.com/dictionary/genotoxin>

IPA: /ˌdʒiː.noʊˈtɔːk.sɪn/

Phonetic Spelling: jee·noh·tok·sin