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Title: Monitoring eIF4F Assembly by Measuring eIF4E-eIF4G Interaction in Live Cells

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **no**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **no**

3. **Filming location:** Will the filming need to take place in multiple locations? **yes**

   If **Yes**, how far apart are the locations? **maybe 100 meters apart from TC room and our bench**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Yuri Frosi:** Deregulation of eIF4F complex signaling is associated with increased translation of specific mRNA subsets involved in cancer proliferation and survival. Here we describe an eIF4E-eIF4G cell-based protein-protein interaction assay that makes it possible to accurately and reliably assess drug induced changes in eIF4F complex integrity in live cells.

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

OPTIONAL:

1.2. **Yuri Frosi:** There is a growing interest in the development of novel modalities that efficiently target protein-protein interfaces. We envision that this eIF4E:eIF4G live cell PPI assay will play a pivotal role in fostering these strategies and validating them.

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

1.3. **Yuri Frosi:** This assay would provide an optimal primary screen for hit to lead optimization of novel eIF4E-eIf4G inhibitors.

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

1.4. **Yuri Frosi:** Seeding cells correctly and performing plasmid transfection are the most challenging aspects of this protocol since they may reflect directly on the expression of the PPI reporter system.

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

Protocol

2. Cell Culture and Transient Transfection

2.1. After thawing and counting cells, seed 6-well plates with HEK 293 cells, using 2 milliliters of standard growth medium per well **[1-TXT]**. *Videographer: This step is important!*

2.1.1. Talent adds cells to 6-well plate. **TEXT: 0.9 - 1.2 x 10^6 cells/well**

2.2. On the morning of day 2, for each planned transfection, dilute 9 microliters of liposome-based solution in a tube containing 125 microliters of reduced serum medium without phenol red **[1]**.

2.2.1. Talent dilutes liposome-based solution with reduced serum medium.

2.3. While the diluted liposomes incubate at room temperature for 5 minutes, prepare the master mix of DNA by diluting 3 micrograms of each plasmid in 125 microliters of reduced serum medium for each transfection tube **[1]**.

2.3.1. Talent dilutes 3 micrograms of plasmid in 125 microliters of medium.

2.4. Add 12 microliters of enhancer reagents to the DNA master mix tube and mix well **[1]**. Immediately add this mixture to each tube of diluted liposomes in a ratio of 1 to 1 **[2]**.

2.4.1. Talent adds enhancer reagents to the DNA master mix tube and mixes well.
2.4.2. Talent adds this mixture to each tube of diluted liposomes.

2.5. After incubating this DNA-lipid complex for 15 minutes at room temperature, add the complex to each well of the 6-well plates **[1]**. Incubate the cells at 37 degrees Celsius with 5 percent carbon dioxide for 24 hours **[2]**.

2.5.1. Talent adds DNA-lipid complex to each well of 6-well plates.
2.5.2. Talent places plates in incubator.

2.6. On the morning of day 3, rinse each well with 1 milliliter of PBS and add 0.3 milliliters of trypsin **[1]**. Incubate the plates at 37 degrees Celsius for 5 minutes **[2]**. *Videographer: This step is important!*

2.6.1. Talent rinses each well with PBS and adds trypsin.
2.6.2. Talent places plates in incubator.

2.7. After incubation, neutralize the trypsin by adding 2 milliliters to each well of the reduced serum medium without phenol red **[1-TXT]**. Transfer the transfected cells into a 15-milliliter tube **[2]**. *Videographer: This step is important!*

2.7.1. Talent adds reduced serum medium to each well. **TEXT: Phenol red interferes with luciferase activity** NOTE: This and next shot together

2.7.2. Talent transfers transfected cells to a 15-milliliter tube.

2.8. Next, centrifuge the cells at 290 times *g* for 5 minutes **[1]**. Aspirate the medium, and resuspend the cell pellet in 2 milliliters of the reduced serum medium **[2]**.

2.8.1. Talent places tube in centrifuge and adjusts settings.
2.8.2. Talent aspirates medium and resuspends cell pellet.

2.9. Seed the transfected HEK 293 cells in 96-well opaque plates, at a density of 30,000 cells per well, in 90 microliters of medium **[1]**. To obtain 3 technical replicates for 3 different compounds within the same experiment, seed 60 wells. Exclude the wells on the edges **[2]**. *Videographer: This step is important!*

2.9.1. Talent adds transfected cells to 96-well plate, in 90 microliters of medium.
2.9.2. Talent continues seeing cells.

2.10. Immediately after seeding the transfected cells, add 10 microliters of 10 percent DMSO compound solution to each well **[1]**.

2.10.1. Talent adds DMSO to each well.

3. Compound Preparation

3.1. Prepare 1 millimolar compound stock solutions by dissolving each compound of interest in 100 percent DMSO **[1]**. In order to have 3 replicates for each compound titration, use 8 microliters of the 1 millimolar compound stock solution **[2]**.

3.1.1. Talent begins dissolving compounds of interest in DMSO.
3.1.2. Talent continues dissolving compounds of interest in DMSO

3.2. Perform a 2-fold serial dilution of each stock compound solution in 8 wells of a 96-well PCR plate by pipetting 4 microliters of the 1 millimolar stock into 4 microliters of 100 percent DMSO for each titration point **[1]**. Discard the excess 4 microliters after the last point of the 2-fold serial dilution **[2]**.

3.2.1. Talent begins serial dilution of stock compounds. Labeled tubes for other dilutions are visible.

3.2.2. Talent discarding the excess 4 microliters.

3.3. Add 36 microliters of HPLC grade sterile water to each tube to prepare 40 microliters of 10 X compound serial dilution solutions in 10 percent DMSO **[1]**. Also prepare a control, 10 percent DMSO-only stock solution in HPLC grade sterile water **[2]**.

3.3.1. Talent adds sterile water to compound serial dilution.
3.3.2. Talent prepares control.

3.4. Add 10 microliters of 10x working solutions to the cells in the 96-well opaque plate in order to yield the intended final concentration with a residual DMSO concentration of 1% **[1-TXT]**. Incubate the plate at 37 degrees Celsius with 5 percent carbon dioxide for 3 hours **[2]**. *Videographer: This step is important!*

3.4.1. Talent adds 10 microliters of working solution to the cells in the 96-well plate.
TEXT: Total volume = 100 µL

3.4.2. Talent places plates in incubator.

4. Luciferase Complementation and Viability Assay

4.1. After 3 hours, start preparing the luciferase substrate reagent by combining 1 volume of substrate with 19 volumes of the dilution reagent **[1]**. *Videographer: This step is important!*

4.1.1. Talent combines 1 volume of substrate with 19 volumes of dilution reagent.

4.2. Use a multichannel pipette to immediately add 25 microliters of the substrate reagent to each well of the 96-well plate with the cells **[1]**. Shake the plate on an orbital shaker at 350 rpm, for 50 minutes at room temperature **[2]**. *Videographer: This step is important!*

4.2.1. Talent uses multichannel pipette to add 25 microliters of substrate reagent.

4.2.2. Talent places plate on orbital shaker, AND/OR plate shaking on orbital shaker.

4.3. To assess luminescence, place the plate on a plate reader. Set the mirror reader to luminescence and the emission filter to 455. Use a measurement height of 6.5 millimeters with a measurement time of 1 second **[1]**.

4.3.1. Talent places plate on plate reader, adjusts settings, and captures measurement.

4.4. To asses cell viability, add 33 microliters of viability assay reagent to each well **[1]**. After 15 minutes at room temperature, assess luminescence with the plate reader by **[2]** setting the mirror reader to luminescence and the emission filter to 600. Use a measurement height of 6.5 millimeters, and a measurement time of 1 second **[3]**. *Videographer: This step is important!*

4.4.1. Talent adds viability assay reagent to each well.

4.4.2. Talent places plate in the plate reader.

4.4.3. Talent adjusts settings on plate reader and captures measurement.

4.5. Use the data from the plate reader to determine the IC50 value of each compound, by curve fitting the data to the 4-parameter fitting curve equation described in the manuscript **[1]**.

4.5.1. Talent at computer begins process of curve fitting.

Results

5. Results: PP242 Disrupts eIF4F Complex Formation

5.1. HEK293 cells were transfected with the eIF4E:eIF4G *(spell out 'E-i-F-4-E-E-i-F-4-G')* complementation system, and then re-seeded and treated with mTOR *(pronounce 'M-tor')* inhibitors **[1]**. When luminescence was assessed four hours after treatment, PP242 and rapamycin both produced a dose-dependent inhibition of the signal **[2]**.

5.1.1. LAB MEDIA: Figure 2.
5.1.2. LAB MEDIA: Figure 2. *Video editor, please emphasize Figure 2A.*

5.2. Neither PP242 nor rapamycin produced a significant decrease in cell viability, indicating that the decrease in luminescence in the eIF4E:eIF4G complementation system is not due to nonspecific cell death but rather to disruption of the eIF4E:4G interaction **[1]**.

5.2.1. LAB MEDIA: Figure 2. *Video editor, please emphasize Figure 2D.*

5.3. Western blot analysis following m-7-GTP pull down experiment showed that 4EBP1-mediated disruption of endogenous eIF4E-eIF4G interaction correlates with the measured eIF4E:eIF4G assay signal **[1]**.

5.3.1. LAB MEDIA: Figure 2. *Video editor, please emphasize Figure 2B and 2C.*

5.4. PP242 was a more potent inhibitor of total 4EBP1 phosphorylation than rapamycin **[1]**. Both inhibitors showed an impact on mTOR signaling normally, with rapamycin being more active against mTORC1 substrates and PP242 targeting both mTORC1 and mTORC2 **[2]**.

5.4.1. LAB MEDIA: Figure 3. *Video editor, please emphasize Figure 3A.*
5.4.2. LAB MEDIA: Figure 3. *Video editor, please emphasize Figure 3B.*

Conclusion

6. Conclusion Interview Statements

6.1. **Yuri Frosi:** The most critical aspects of this procedure are correct cell seeding on the day of transfection, reseeding the cells in medium without phenol red, assessing luminescence for the eIF4E-4G complementation assay, and running the viability assay on the same plate.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.9.1, 4.3.1, 4.4.1.*

6.2. **Yuri Frosi:** A secondary viability assay ca be performed to asses any drug off target and non-specific effects.

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