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

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30th September 2019

Dear Editor

Please find the manuscript enclosed entitled 'Monitoring eIF4F Assembly by Measuring eIF4E-eIF4G Interaction in Live Cells ' that we would like to be considered for publication in JoVE. Enclosed below is the original cover letter.

In this work we have developed an innovative live cell protein-protein interaction (PPI) assay, using NanoBit technology (PROMEGA), which measures the eIF4E:eIF4G interaction and allows activity of the intracellular eIF4F complex to be quantitatively evaluated. eIF4F activity is frequently elevated in many cancers, usually as a direct consequence of commonly occurring cancer mutations that can be found in signal transduction pathways that feed into the translational machinery such as RAS/ERK e.g. RAS^{G12D}, $B\text{-}RAF^{V600E}$ and PI3K/AKT e.g. AKT^{E17K}, $PI3K^{E542K}$. Hyper-activation of the eIF4F complex as a result of these mutations results in increased protein translation of a large group of proteins involved in oncogenic development (MCL-1, C-MYC and VEGF). Regulation of the eIF4F complex by both these pathways is primarily through MNK mediated phosphorylation of eIF4E and mTORC1 mediated phosphorylation of 4E-BP1, respectively. Non phosphorylated 4E-BP1 prevents eIF4F complex formation through binding eIF4E directly and inhibiting its interaction with eIF4G. Using well known inhibitors of mTORC1 we showed that the eIF4E:4G live cell PPI assay could accurately detect the release of endogenously un-phosphorylated 4E-BP1. Additionally we could also differentiate these inhibitors in terms of potency and demonstrate the increased effectiveness of the ATP active site inhibitors, P242, over allosteric inhibitors (e.g. Rapamycin).

We envision that the eIF4E:4G live cell PPI assay could be a critical tool in identifying novel modalities that inhibit this interaction and in isolating new leads for therapeutic development programs.

Kind regards

Christopher Brown

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TITLE:

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

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KEYWORDS:

Cellular Biology, eIF4F complex, Translational control, Luciferase complementation assay, mTOR signaling, Protein-protein interactions

SUMMARY:

Here, we present a protocol to measure eIF4E-eIF4G interaction in live cells that would enable the user to evaluate drug induced perturbation of eIF4F complex dynamics in screening formats.

ABSTRACT:

Formation of the eIF4F complex has been shown to be the key downstream node for the convergence of the signaling pathway that often undergoes oncogenic activation in humans. eIF4F is a cap-binding complex involved in the mRNA-ribosome recruitment phase of translation initiation. In many cellular and pre-clinical model of cancers, the deregulation of eIF4F leads to increased translation of specific mRNA subsets that are involved in cancer proliferation and survival. eIF4F is a hetero-trimeric complex built from the cap-binding subunit eIF4E, the helicase eIF4A and the scaffolding subunit eIF4G. Critical for the assembly of active eIF4F complexes is the protein-protein interaction between eIF4E and eIF4G proteins. In this article, we describe a protocol to measure eIF4F assembly that monitors the status of eIF4E-eIF4G interaction in live cells. The eIF4e:4G cell-based protein-protein interaction assay also allows drug induced changes in eIF4F complex integrity to be accurately and reliably assessed. We envision that this method can be applied for verifying the activity of commercially available compounds or for further screening of novel compounds or modalities that efficiently disrupt formation of eIF4F complex.

INTRODUCTION:

Control of gene expression plays a pivotal role in the correct execution of cellular programs such as growth proliferation and differentiation. A regulatory control mechanism can be exerted either at the level of gene transcription or at the level of mRNA translation. In the last decade, it has become increasingly evident that translational control by modulation of the initiation process

rather than the later steps of elongation and termination can finely regulate synthesis of specific subsets of proteins that play a wide range of biological functions.

Increased translation of mRNAs involved in survival, anti-autophagic and anti-apoptotic responses have been implicated in several cancers and have also been causatively linked to either aberrant activation or over expression of translation initiation factors[1].

The eIF4F complex is a master regulator of translation initiation. By binding the cap-structure on the 5' end of mRNAs, eIF4F is driving initial mRNA-ribosome recruitment and in turn increasing mRNA translation efficiency of weakly translated eukaryotic mRNAs[2]. eIF4F mediated translation of cancer-related mRNAs has been reported for many cancer models harboring aberrant activation of RAS/MAPK or AKT/TOR pathways, suggesting that cancer cells upregulate eIF4F to boost their own pro-neoplastic activity. Disruption of this feed-forward loop by inhibiting eIF4F complex formation is thereby a very promising therapeutic strategy[3,4].

The eIF4F complex consists of (i) eIF4E, the cap-binding subunit of eIF4F that interacts with the cap structure found at the 5' UTR of mRNA, (ii) eIF4A, the RNA helicase and (iii) eIF4G, the scaffold protein that interacts with both eIF4A and eIF4E and eventually recruits the 40S ribosomal subunit[5]. eIF4G association with eIF4E is the rate-limiting step for the assembly of functional eIF4F complexes and it is negatively regulated by the eIF4E binding proteins (4EBPs)[6]. By competing with eIF4G binding to eIF4E through an interface that consists of canonical and non-canonical eIF4E binding sequences[7-9] (region spanning aa 604-646 on human eIF4E), 4EBP reduces the pool of eIF4E actively involved in translation and preventing eIF4F complex formation. Interplay of these protein-protein interactions is mainly regulated by the mammalian target of rapamycin (mTOR)-mediated phosphorylation of 4EBP. Upon mitogenic stimuli, mTOR directly phosphorylates the members of the 4E-BP protein family, decreasing their association with eIF4E and, thereby, promoting eIF4E-eIF4G interaction and formation of functional eIF4F complexes[10].

Despite the great effort in developing compounds targeting eIF4F complex integrity, the lack of assays measuring direct disruption of eIF4E-eIF4G interaction in live cells has limited the search for cellular active hit compounds. We have applied a luciferase assay based on a coelenterazine analog (e.g., Nanoluc-based complementation assay) to monitor in real time the status of eIF4F integrity through the eIF4E-eIF4G interaction. The luciferase complementation protein system consists of an 18 kDa protein fragment (SubA) and 11 amino acid peptide fragment (SubB) optimized for minimal self-association and stability[11]. Once expressed as a fusion product with the human full length eIF4E and the eIF4E interaction domain from human eiF4G 1 (aa 604-646), the two interacting proteins will bring the SubA and SubB fragment into close proximity of each other and will induce the formation of the active luciferase that, in presence of a cell permeable substrate, will eventually generate a bright luminescent signal (**Figure 1**). We have reported elsewhere the construction and validation of the eIF4E:eIF4G$^{604-646}$ complementation system.

Here, we describe how the eIF4E:eIF4G$^{604-646}$ complementation system (available upon request) can be applied to accurately measure 4EBP1-mediated eIF4E-eIF4G disruption in live cells.

Additionally, we demonstrate its utility by measuring the effects of several mTOR inhibitors that are currently under clinical trials as potential cancer therapeutic drugs[12]. Because off-targets effects often mask drug-specific activity, we also describe how the versatility of the eIF4E:eIF4G$^{604-646}$ system measurement can be extended with orthogonal measurements of cellular viability to take these into account.

PROTOCOL:

HEK293 cell line was used for the protocol and was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin. Cells were cultured at 37 °C with 5% CO_2 in a humidified environment.

1. Quantitative assessment of eIF4F complex disruption via eIF4E:eIF4G$^{604-646}$ complementation assay

1.1 Cell culture and transient transfection of eIF4E:eIF4G$^{604-646}$ complementation assay

1.1.1 Use freshly thawed cells with less than 20 passages for all experiments. On day 1, determine the cell number using a standard cell counter and count the total viable cells using the Trypan blue exclusion method[13].

1.1.2 Seed 6-well plates with 0.9 - 1.2 x 10^6 of HEK 293 cells per well in 2 mL of standard growth medium.

NOTE: In order to achieve the best transfection efficiency within the cell lines indicated above, ensure that the plated cells are 70-90% confluent the day after seeding.

1.1.3 On the morning of day 2, co-transfect cells with SubA-eIF4E and eIF4G$^{604-646}$-SubB plasmid using a lipid-based transfection reagent (see **Table of Materials**) as described below.

1.1.3.1 Dilute 9 µL of liposome-based solution in a tube containing 125 µL of reduced serum medium without phenol red (see **Table of Materials**) for each transfection and incubate at room temperature for 5 min.

1.1.3.2 Prepare master mix of DNA by diluting 3 µg of each plasmid in 125 µL of reduced serum medium for each transfection tube.

1.1.3.3. Add 12 µL of enhancer reagents to the DNA master mix tube, mix well and immediately add the DNA: enhancer master mix to each tube of diluted liposomes in a ratio 1:1. Incubate for 15 min at room temperature.

1.1.3.4. Add the DNA-lipid complex to each well and incubate the cells in a 37 ˚C incubator with 5% CO_2 for 24 h.

1.1.4 On the morning of day 3, rinse each well with 1 mL of PBS.

1.1.5 Remove the PBS and incubate cells in each well with 0.3 mL of trypsin for 5 min at 37 ˚C.

1.1.6 Neutralize trypsin by adding 2 mL of the reduced serum medium without phenol red containing 0% FCS in each well and transfer transfected cells into a 15 mL tube.

NOTE: Phenol red can interfere with the luciferase activity; therefore, cells must be handled from this step onward in medium without phenol red.

1.1.7 Spin down cells for 5 min at 290 *x g* and aspirate the medium. Resuspend the cells in 2 mL of the reduced serum medium without phenol red containing 0% FCS. Count as described in step 1.1.

1.1.8 Seed transfected HEK 293 cells in 96 well opaque plates at a density of 30,000 cells per well in 90 µL of medium without phenol red containing 0% FCS. In order to obtain 3 technical replicates within the same experiment for 3 different compounds, seed 60 wells of the plates with cells excluding the wells on the edges.

1.1.9 Immediately after seeding the transfected cells, add 10 µL of 10% DMSO compound solution (see step 1.2).

1.2 Compound preparation

1.2.1 Prepare 1 mM compound stock solutions by dissolving the compound of interest in 100% DMSO (v/v). In order to have 3 replicates for each compound titration, use 8 µL of the 1 mM compound stock solution.

NOTE: The volume of 1 mM stock compound used is more than what it is needed. This is done to take into account pipetting errors if any.

1.2.2 Perform a 2-fold serial dilution of the stock compound solution with 4 µL of the 1 mM stock into 4 µL of 100% DMSO for each titration point.

NOTE: For a complete compound titration, perform 9 serial dilutions from the starting stock in 100% DMSO. If multiple compounds need to be tested, use a 96 well plate and a multichannel pipette to facilitate this step and subsequent dilutions.

1.2.3 Add 36 µL of HPLC grade sterile water for each point of the 2-fold serial dilution to prepare a 40 µL of 10x working solutions in 10% DMSO (v/v). As for treatment control, also prepare a 10% DMSO only stock solution in HPLC grade sterile water (i.e., 100 µM to 0.39 µM 10% DMSO stock series will lead to a final solution of 10 µM to 0.039 µM, respectively in 1% DMSO when added to the cells).

1.2.4 Add 10 µL 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% (v/v) in a total volume of 100 µL and incubate for 3 h at 37 ˚C with 5% v/v atmospheric CO_2.

1.3 Luciferase complementation and viability assay

1.3.1 After 3 h of drugging, start preparing the luciferase substrate reagent by combining 1 volume of substrate with 19 volumes of the dilution reagent (see manufacturer's instructions).

NOTE: The luciferase assay is performed using a commercially available kit (see **Table of Materials**).

1.3.2 Use a multichannel pipette to immediately add 25 µL of substrate reagent.

1.3.3 Shake the plate at 350 rpm for 50 min on an orbital shaker at room temperature.

1.3.4 Assess luminescence using a plate reader. To do so, set the mirror reader on luminescence and the emission filter on 455. Use a measurement height of 6.5 mm with a measurement time of 1 s.

1.3.5 In order to asses cell viability, add 33 µL of viability assay reagent and then re-measure luminescence again after 15 min at room temperature, using the plate reader.

1.3.6 Assess luminescence with the plate reader by setting the mirror reader on Luminescence and the emission filter on 600. Use a measurement height of 6.5 mm with a measurement time of 1 s.

NOTE: Viability is assessed by measuring the intracellular level of ATP upon cell lysis as per manufacturer instruction. Multiplexing is possible in this case since the viability assay uses a different luciferase with a different emission wavelength.

1.3.7 Use the data to determine the IC50 value of each compound by curve fitting the data to 4 parameter fitting curve equation:

Y= ((A-D)/(1+((x/C)^B))) + D.

The numerical values that D and A must be constrained to in the curve fitting are:
D = luminescent value on the Y-axis for minimal curve asymptote or minimal theoretical level of response expected from the eIF4E:4G complementation assay.
A = luminescent value on the Y-axis for maximal curve asymptote or maximal theoretical level response expected from the eIF4E:4G complementation assay.

NOTE: The value for the minimal response is derived from the 1% DMSO (v/v) control treatment and the value for the maximal response is derived from the maximal response of a high affinity

mTOR inhibitor that has plateaued with no effect on cell viability.

2. Correlating eIF4E:eIF4G$^{604-646}$ asssay inhibition with eIF4F complex disruption in cells

2.1 To confirm that the signal being measured by the assay corresponds to the physical disruption of eIF4E-eIF4G interaction by the compound, seed cells as described in step 1.1.

2.2 The day after, replace the medium with 1 mL of reduced serum medium not containing phenol red and incubate for 4 h with the compound of interest. Ensure residual DMSO concentration of 1% (v/v) in a total volume of 1 mL.

NOTE: In order to easily allow a correlation with the result, use compound concentrations at the beginning, midpoint and endpoint of the measured complementation assay titration curve.

2.3 Lyse cells and performed m^{7}GTP pull down to isolate eIF4F and eIF4E:4EBP1 complexes. Detailed procedures on how to perform the m^{7}GTP pull down experiment can be found elsewhere[14].

2.4 Detect m^{7}GTP bound eIF4G, eIF4E and 4EBP1 protein levels by western blot analysis, and then correlate to eIF4F complex disruption with complementation assay signal inhibition.

REPRESENTATIVE RESULTS:

In order to validate the sensitivity of the eIF4E:eIF4G$^{604-646}$ complementation system, 4EBP1 mediated inhibition of eIF4F complex assembly was assessed by using mTOR inhibitors. By inhibiting mTORC1 kinase dependent phosphorylation of the 4EBP protein family, mTOR inhibition enhances 4EBP1 association to eIF4E and, therefore, eIF4F disassembly[15]. Two classes of mechanistically different inhibitor of mTOR kinases were tested: rapalogs (e.g., Rapamycin) that are allosteric inhibitors of mTORC1 but not mTORC2 and ATP competitive-based inhibitors (e.g., PP242) that are designed to specifically inhibits both mTORC1 and mTORC2 kinases catalytic activity.

HEK293 cells were transfected with the eIF4E:eIF4G$^{604-646}$ complementation system as described in step 1. After 24 h of transfection, cells were re-seeded and treated with the mTOR inhibitors PP242 and rapamycin (as described in step 1.2). Four hours after the treatment, luminescence was assessed, as described previously, followed by cell viability. As shown in **Figure 2**, PP242 produces a dose-dependent inhibition of the signal with a calculated IC50 of 0.72 ± 0.04 µM, while an IC50 of 6.88 ± 0.88 µM is derived for rapamycin (**Figure 2A**). Plates were then multiplexed for cellular viability assay (**Figure 2D**). This analysis shows that neither PP242 nor rapamycin produces a significant decrease in cell viability, proving that the decrease in luminescence in the eIF4E:eIF4G$^{604-646}$ complementation system is not due to nonspecific cell death but rather through disruption of the eIF4E:4G interaction.

A m^{7}GTP pull down experiment performed by incubating untransfected cells with compound concentrations that correspond to the beginning, mid and end points of the measured titration

curve in **Figure 2A** show that 4EBP1-mediated disruption of endogenous eIF4E-eIF4G interaction correlates with the measured eIF4E:eIF4G$^{604\text{-}646}$ assay signal (**Figure 2B, 2D**). Consistent with these results, PP242 is shown to be a more potent inhibitor of total 4EBP1 phosphorylation than rapamycin under the experimental conditions tested in HEK 293 cells (**Figure 3A**), while both inhibitors showed an impact to mTOR signaling normally, with rapamycin being more active against mTORC1 substrates and PP242 targeting both mTORC1 and mTORC2 (**Figure 3B**).

Taken together, these results showed that PP242 is more effective in disrupting eIF4F complex formation than rapamycin in HEK293 cells and further demonstrate that the eIF4E:eIF4G$^{604\text{-}646}$ system can accurately measure eIF4F complex assembly in living cells.

FIGURE AND TABLE LEGENDS:

Figure 1: eIF4E:eIF4G$^{604\text{-}646}$ complementation system. Schematic representation showing how the interaction of protein X (eIF4E) and protein Y (eIF4G$^{604\text{-}646}$) enables SubA and SubB fusions to get in close proximity and reconstitute the active luciferase.

Figure 2: 4EBP1-mediated disruption of eIF4F complex. (**A**) PP242 and rapamycin eIF4E:eIF4G$^{604\text{-}646}$ assay titration modelling the interaction between eIF4E and eIF4G in transfected HEK 293 cells. (**B,C**) Western blot analysis showing endogenous level of eIF4E, eIF4G and 4EBP1 in HEK 293 extracts and in m7GTP pull down after incubation of cultured cells with different concentration of PP242 or Rapamycin respectively. (**D**) Treated cells in A where multiplexed for cell viability and luminescence assessed. All values represent mean ± SD (n=3). This figure has been modified from[16].

Figure 3: Differential effect of mTOR inhibition on 4EBP1 phoshorylation (**A**) Western blot analysis of phosphorylation status of 4EBP1 in non-transfected HEK293 cells treated with indicated concentration of PP242 and rapamycin. (**B**) Western blot analysis of AKT and S6 phosphorylation status in non-transfected HEK293 cells treated with either the dual MTORC1/2 active site inhibitors PP242, or the allosteric inhibitor of mTORC1 Rapamycin. Beta actin was visualized for loading control as well as total 4EBP1, AKT and S6. This figure has been modified from[16].

DISCUSSION:

The method described in this article utilizes a luciferase-based complementation assay to quantitatively monitor eIF4F complex assembly through direct measurement of eIF4G-eIF4E interaction in live cells. We provide details for use of eIF4E-eIF4G complementation system and we also showed that the system is extremely accurate in measuring drug-induced 4EBP1-mediated dissociation of eIF4E-eIF4G interaction[16]. In order to facilitate the throughput of the assay, the experimental setup described in this article has been designed for a 96 well microplate format usage.

For optimal results, two critical steps should be considered when performing the assay. First, the transfection efficiency between experiments should remain similar. This can be ensured through the use of low passage number cells, and by rigorously counting cells on the day of seeding. Cell

confluency should also be assessed before DNA transfection is carried out, as it is not recommended to transfect cells with lipid-based transfection reagent if cell confluency is less than 70-90%. Second, it is important to multiplex the complementation assay with a cell viability assay. Some compounds may impact the luciferase signal primarily by decreasing the number of viable cells through deleterious effects. It is, therefore, important to measure the viability of the cell immediately after the eIF4E:eIF4G$^{604-646}$ complementation assay to address off-target and non-specific effects.

Protein-protein interfaces, such the one between eIF4E and eIF4G, that are devoid of hydrophobic clefts and are relatively large and planar, are generally considered to be "un-druggable" by conventional small molecule therapeutics (<500 MW)[17]. Thus, there is a growing interest in the development of novel modalities that efficiently interact with these type of surfaces (e.g., macrocyclic and peptidomimetic compounds). However, many of these novel modalities are not innately able to cross the cell membrane and engage their target. To circumvent these issues, many research groups are conducting research into new chemical optimization and cellular delivery strategies. We envision that the eIF4E:eIF4G$^{604-646}$ live cell PPI assay and other similar PPI derived assays will play a pivotal role in fostering these strategies and validating them.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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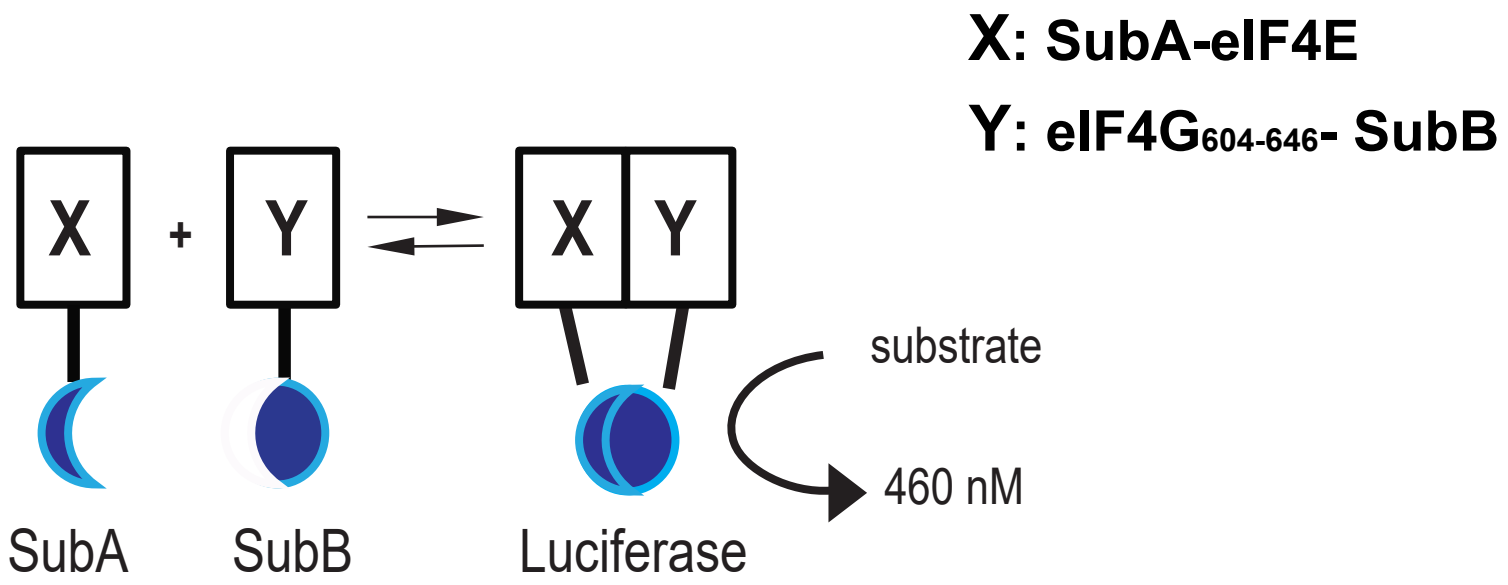

Figure

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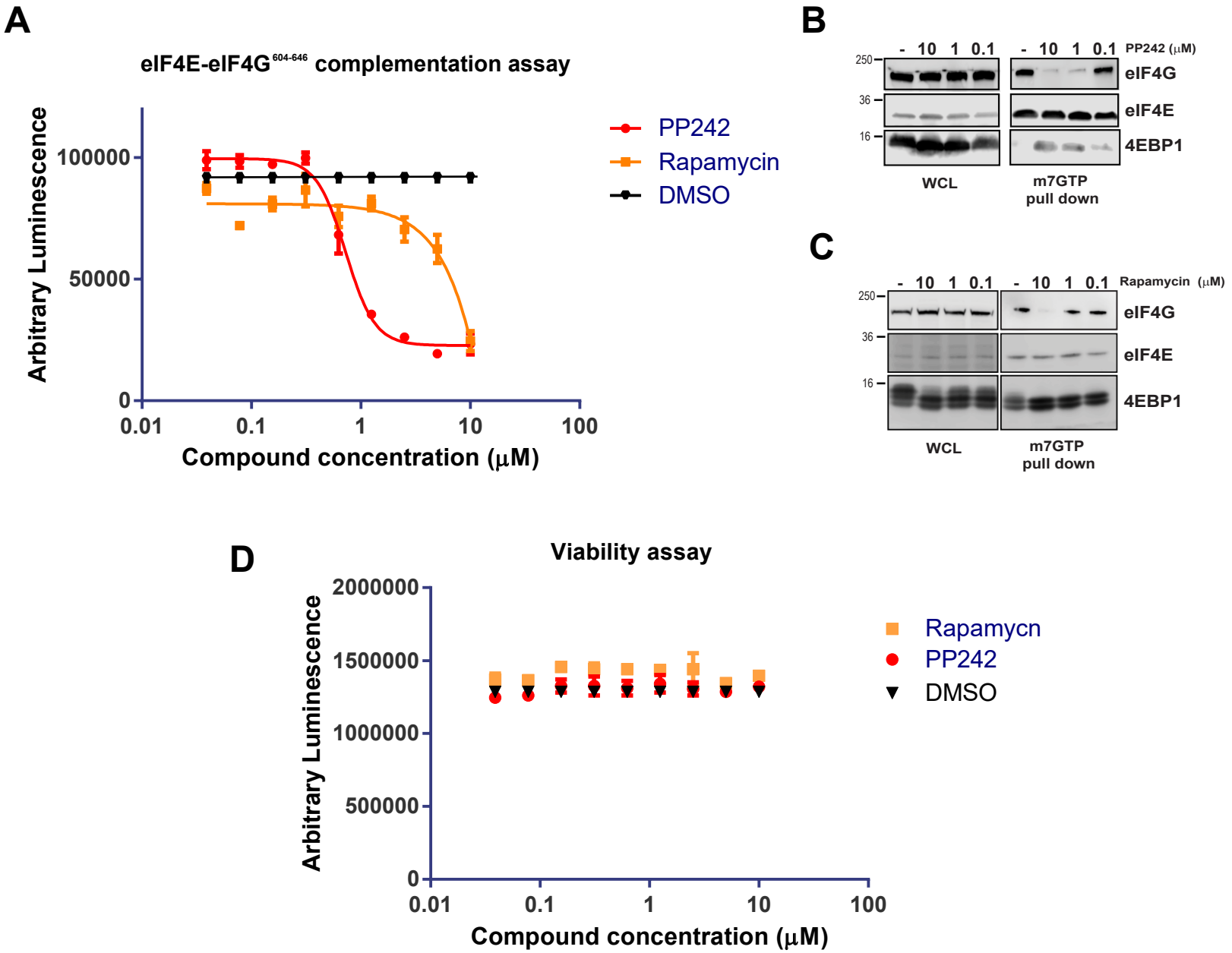

Figure

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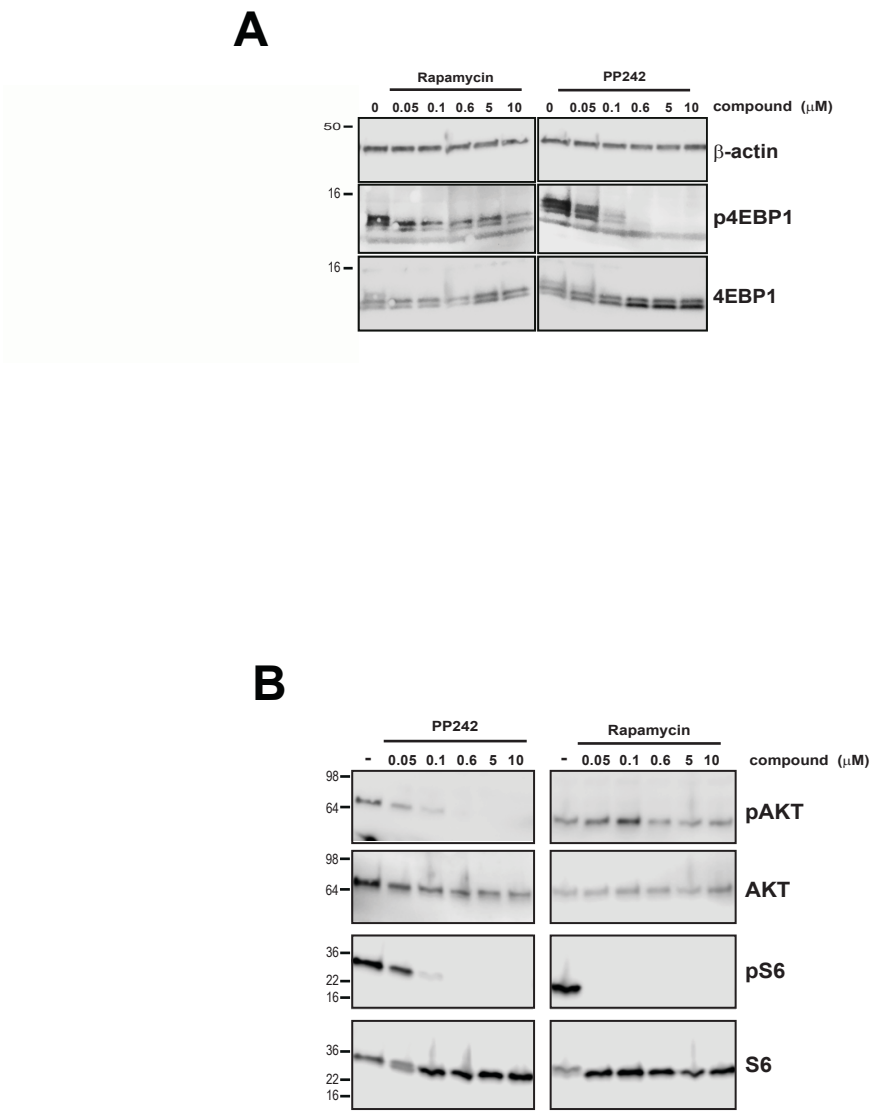

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
293FT cells	Thermo Fisher Scientific	R70007	
Cell culture microplate 96 well , F-Bottom	greiner bio-one	655083	
Cell titer Glo 2.0	PROMEGA	G9241	
Envision Multilabel Reader	PerkinElmer	not applcable	
Finnpipette F2 Multichannel Pipett	Thermo Fisher Scientific	4662070	
Finnpipette F2 Multichannel Pipett	Thermo Fisher Scientific	4662050	
FUGENE6	PROMEGA	E2692	
Lipofectamine 3000	Thermo Fisher Scientific	L3000015	
NanoBiT PPI Starter Systems	PROMEGA	N2014	
Optimem I Reduced Serum Mediun, no phenol red	Thermo Fisher Scientific	11058021	
Orbital shaker	Eppendorf	not appicable	
γ-Aminophenyl-m^7GTP (C_{10}-spacer)	Jena Bioscience	AC-155S	

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25th November 2019

Dear Editor

We would like to thank the reviewers for reading and critically evaluating our manuscript. Please find enclosed the updated version of the manuscript entitled '**A new valuable tool to monitor eIF4F assembly by measuring eIF4E-eIF4G interaction in live cells**" I have outlined below our replies and changes we have made within the manuscript to the comments made by the reviewers. I have also modified the main text according to the Editor suggestions. SmBIT and LgBIT are now SmallBit and BigBit respectively while NanoBit as been replaced by eIF4E-eIF4G$^{604-646}$ complementation assay

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The protocol described in the paper entitled "A new valuable tool to measure protein-protein interactions of eIF4F assembly in live cells" by Yuri Frosi et al., aims to provide a new method to measure the eIF4E-eIF4G protein interaction in live cells.
The strategy introduced by this paper is to follow the interaction of two components of the pre-initiation complex eIF4F by luminescence. The authors use the NanoBiT complementation reporter by fusing the Small and Large BiT to the eIF4E and eIF4G604-646 peptide respectively, allowing to correlate the luminescent signal provided by proximity of the split reporters to protein-protein interaction.
Because of the sensitivity and simplicity of this assay, it represents an useful technical advancement with interesting potential applicability for the screening of inhibitors of the pre-initiation complex assembly.
However, the work should be strengthened in some points in order to provide a solid assay for the users.

Major Concerns:
1. The system used by the authors is based on the use of the NanoBiT complementation reporter but a clear explanation of this system should be included. A scheme of the combinatorial fusion proteins would be very helpful to rationalize the generation of luminiscent signal.

We thank referee for his/her comment. The scheme has been included as Figure 1 and full explanation has been supplied in both Representative Result and Figure legend 1

2. The authors constructed the eIF4G-LgBiT fusion protein using only the eIF4G604-646 domain. It would be informative to include the rationale of this choice and the reference works that describe the eIF4G604-646 binding properties to eIF4E.

We thank referee for his/her comment. The rationale of this choice has been included in the introduction :

By competing with eIF4G binding to eIF4E through an interface that consists of canonical and non-canonical eIF4E binding sequences[7,8,9](region spanning aa 604-646 on human eIF4E) 4EBP reduces the pool of eIF4E actively involved in translation and preventing eIF4F complex formation.

And in The Representative Result:

Once expressed as a fusion product with the the human full length eIF4E and the eIF4E interaction domain from eiF4G (aa 604-646), the two interacting proteins will bring the Big BiT and Small Bit into close proximity of each other and will induce the formation of the active luciferase that, in presence of a cell permeable substrate, will eventually generate a bright luminescent signal (Figure 1)

3. In paragraph 1.1, the authors highlight the importance of keeping the length of the linker between NanoBiT tags and eIF4E and eIF4G the same. However, there is no description or graphical representation of the construct that would facilitate the reader to better understand the reporter system. This should be included.

We thank Referee 1 for this comment. We have removed this part and modified the text as following:

We have applied a Nanoluc-based complementation assay to monitor in real time the status of eIF4F integrity through the eIF4E-eIF4G interaction. Here we describe how the eIF4E:eIF4G$^{604-646}$ complementation system (available upon request) can be applied to accurately measure 4EBP1-mediated eIF4E-eIF4G disruption.

4. In order to correlate the luciferase signal observed in figure 1 (eIF4G-LgBIT+ SmBIT-eIF4E) with the interaction between eIF4G604-646 and eIF4E we suggest performing a classical Co-IP of the reporter construct in the same conditions tested. This reviewer anticipates an increased binding of SmBIT-4E with the endogenous 4EBP upon mTOR inhibition. Moreover, it is important to determine the partnership extent with the endogenous eIF4E and eIF4G respectively.

We thank Referee 1 for this comment. Validation was already published by our lab (please see REF 14) and we also claim it in the Representative result part:

We have reported elsewhere the construction and validation of the eIF4E:eIF4G$^{604-646}$ complementation system. Here we describe the protocol to accurately quantify eIF4F complex formation by measuring the eIF4E-eIF4G interaction with the eIF4E:eIF4G$^{604-646}$ complementation assay in living cells.

5. The experiments shown in this protocol are done after 4h of treatment with mTOR inhibitors. The authors should specify if this assay can be used for longer treatments (for instance during 24 or 48h), and the controls needed in this case (expression of the reporters, etc).

We thank Referee 1 for this comment. We have now included this note in paragraph 1.8:

NOTE: longer time incubation for each drug can be tested without affecting reporter sensitivity

6. The authors didn't verify the mTOR signaling in the experimental conditions tested, hence it is difficult to extrapolate the efficacy of mTOR inhibitions and correlate it with the reporter activity. Moreover, it is particularly important to know if the expression of the reporter does compete with endogenous 4E-BPs.

We thank Referee 1 for his/her comment. We have now included Figure 3 and the following part to verify mTOR signalling inhibition under experimental condition tested in complementation assay and pull down experiments:

Consistently with these results, PP242 resulted to be a more potent inhibitor of total 4EBP1 phosphorylation than Rapamycin under the experimental conditions tested in HEK 293 cells (Figure 3A), while both inhibitors showed to impact mTOR signaling normally, with Rapamycin being more active against mTORC1 substrates and PP242 targeting both mTORC1 and mTORC2 (Figure 3B).

7. For the validation of the system, the mTOR allosteric and ATP-site inhibitors, rapamycin and PP242 respectively, are used as positive controls of the disruption of the pre-initiation complex. Specific inhibitors of the eIF4E and eIF4G interaction, like 4EGI-1 compound (Sekiyama et al. PNAS, 2015), would constitute a gold standard control in blocking this association.

We appreciate Referee 1 comment on this. 4EGI-1 data can be found in our previous publication (Ref 14) and it is in perfect agreement with what Referee 1 suggested. However in this article we have also show that 4EGI-1 is affecting viability of transfected HEK293 cells over 4 hrs therefore limiting the correct interpretation of the results. On the contrary mTOR inhibitors don t show any non-specific inhibition of luciferase signal under experimental conditions have been tested.

8. The authors propose to the user to generate and test the different combinations of constructs (1-NanoBit eIF4E:eIF4G604-646 Construction Experiment). However they already verified the optimal combination (eIF4G-LgBIT-smBIT-eIF4E). Therefore the protocol would be more straightforward if the authors provide the eIF4G-LgBIT and smBIT-eIF4E plasmids (deposited on Addgene or available upon request), and move the results of the construction experiment in the "representative results".

Thanks Referee 1 for this comment. We have already reply to this in Referee 1 point 3

9. The title "A new valuable tool to measure protein-protein interactions of eIF4F assembly in live cells" is overstated since the method measures the interaction between eIF4G and eIF4E and not all the components of the eIF4F complex.

We thanks referee 1 his/her comment. We have now change the title as following:

A new valuable tool to monitor eIF4F assembly by measuring eIF4E-eIF4G interaction in live cells

Minor Concerns:
1. For the construct experiment the authors used Fugene reagent for cell transfection, however in the quantitative assessment they recommend Lipofectamine 3000. This discrepancy needs to be clarified.

We thanks referee 1 his/her comment. We have removed this part

2. In the description of the Quantitative assessment in the step 2 the authors specify to use media without red phenol. Nevertheless, in step 3 they specify to resuspend the pellet in Opti-mem in order to dilute the residual trypsin and the red phenol. Paradoxical the Optimem contains red phenol as stated: "In order to dilute the residual trypsin and red phenol, resuspend the pellet in opti-mem with 0% FCS (containing red phenol)". Moreover, across the protocol in some steps the media include red phenol and in others not. It will be important to explain if and how the red phenol could affect the assay.

We thanks referee 1 his/her comment. We have corrected the paragrah and add a note explaining why we are using optimem withour red phenol as following:

On the morning of day 3, trypsinize and pool together transfected cells into a 15 ml falcon tube. Spin down cells for 5 min at 290 rcf and aspirate medium. In order to dilute the residual trypsin, resuspend the pellet in opti-mem with 0% FCS (no red phenol) and estimate cell number as described above.

Note: Red phenol can interfere with the luciferase activity, therefore cells have to be re-seeded in Optimem without red-phenol.

Reviewer #2:
This manuscript describes a protocol that will be of interest to many scientists. The method developed by the authors is very useful, and informative and the he manuscript is clearly written. There are some errors/omissions that the authors should correct to improve the quality.

1. There are three eIF4G paralogs, please clearly indicate which one(s) are referred throughout the text.

We thanks referee 2 his/her comment. We have now modified the text in the Representative result part as following:

Once expressed as a fusion product with the the human full length eIF4E and the eIF4E interaction domain from human eiF4G 1 (aa 604-646), the two interacting proteins will bring the Large BiT and Small Bit into close proximity of each other and will induce the formation of the active luciferase that, in presence of a cell permeable substrate, will eventually generate a bright luminescent signal (Figure 1).

2. p. 1, line 54. "m7GTP cap structure". m7GTP is part of the cap structure, the full cap structure is m7GpppN.

We thanks referee 2 his/her comment. We have now modified the text in the Representative result part as following:

The eIF4F complex consists of (i) eIF4E, the cap-binding subunit of eIF4F that interacts with the cap structure found at the 5' UTR of mRNA, (ii) eIF4A, the RNA helicase and (iii) eIF4G, the scaffold protein that interacts with both eIF4A and eIF4E and eventually recruits 40S ribosomal subunit[5]

3. p. 1, line 63. Up to 8 different phosphorylation sites on 4E-BP have been identified - it is very unlikely that mTOR phosphorylates all of them, based on the literature. Thus, the authors should rephrase their statement.

We thanks referee 2 his/her comment. We have now rephrase the text as following:

Upon mitogenic stimuli mTOR directly phosphorylates the members of the 4E-BP protein family, decreasing their association with eIF4E and thereby promoting eIF4E-eIF4G interaction and formation of functional eIF4F complexes[10,11]

4. p.2, Line 89. This part could use a diagram to help the reader: (i) understand exactly how the fusions look, and (ii) know what the optimal linker length between NanoBit tags and 4E or 4G should be (line 94). Also, it is not clear why the authors are suggesting the reader to generate all possible combinations N- and C-terminal 4E and 4G fusions. Presumably, they already know which one gives the best signal-to-noise response in their hands.

We thanks referee 2 his/her comment. We have now removed the part describing plasmid construction and clearly stated that plasmids will be provided upon request as following:

We have applied a Nanoluc-based complementation assay to monitor in real time the status of eIF4F integrity through the eIF4E-eIF4G interaction. Here we describe how the eIF4E:eIF4G$^{604-646}$ complementation system (available upon request) can be applied to accurately measure 4EBP1-mediated eIF4E-eIF4G disruption

Referee 2 can alo find a scheme in Figure 1 showing how our eIF4E-eIF4G complementation system works

5. p.2, line 20. The authors state that they fused eIF4G amino acids 604-646 to SmBit. Please provide the NCBI reference for this sequence as there are multiple eIF4G1 isoforms.

We are using human Isoform A (identifier: **Q04637-1**).

6. p.3, line 134. "eIF4G-LgBit plasmid" is incorrect. The authors have fused amino acids 604-646 to LgBit. This should be rephrased.

We have now corrected as following:

On the morning of day 2, co-transfect cells with 3 µg of SmallBit-eIF4E and eIF4G$^{604-646}$-Bigbit plasmid using Lipofectamine according to manufacturer's instructions and incubate again cells at 37˚C 5% CO_2.

7. p.3, line140 "290 rcf"; shouldn't this be "290 x g"?

We have now replaced 290 rcf with 290 x g

8. p.4, line 193. M7GTP [pull down will also isolated eIF4E:4EBP complexes, not only eIF4F.

Please refers to the following part in the protocol were we are describing all species being pulled down:

2.2 *Lyse cells and performed m^{7}GTP pull down to isolate eIF4F and eIF4E:4EBP1 complexes. Detailed procedures on how to perform the m^{7}GTP pull down experiment can be found elsewhere[16]*

2.3 *Detect m^{7}GTP bound eIF4G, eIF4E and 4EBP1 protein levels by western blot analysis, and then correlate to eIF4F complex disruption with complementation assay signal inhibition*

9. Table of Materials. What is the source of m7GTP-agarose?

We have now included the source: γ-Aminophenyl-m^{7}GTP (C_{10}-spacer)-Agarose from Jena Bioscience

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Author(s): Yuri Frost, Siti Radiah Rasley, Christopher J. Brown

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