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TITLE:

Effect of Fluorescent Proteins on Fusion Partners Using Polyglutamine Toxicity Assays in Yeast

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KEYWORDS:

Fluorescent proteins, polyglutamine toxicity, yeast growth assays, aggregation, green fluorescent protein, fluorescent microscopy

SUMMARY:

This article describes protocols to assess the effect of fluorescent proteins on the aggregation and toxicity of misfolded polyglutamine expansion for the rapid evaluation of a newly uncharacterized fluorescent protein in the context of fluorescent reporters.

ABSTRACT:

For the investigation of protein localization and trafficking using live cell imaging, researchers often rely on fusing their protein of interest to a fluorescent reporter. The constantly evolving list of genetically encoded fluorescent proteins (FPs) presents users with several alternatives when it comes to fluorescent fusion design. Each FP has specific optical and biophysical properties that can affect the biochemical, cellular, and functional properties of the resulting fluorescent fusions. For instance, several FPs tend to form nonspecific oligomers that are susceptible to impede on the function of the fusion partner. Unfortunately, only a few methods exist to test the impact of FPs on the behavior of the fluorescent reporter. Here, we describe a simple method that enables the rapid assessment of the impact of FPs using polyglutamine (polyQ) toxicity assays in the budding yeast *Saccharomyces cerevisiae*. PolyQ-expanded huntingtin proteins are associated with the onset of Huntington's disease (HD), where the expanded huntingtin aggregates into toxic oligomers and inclusion bodies. The aggregation and toxicity of polyQ expansions in yeast are highly dependent on the sequences flanking the polyQ region, including the presence of fluorescent tags, thus providing an ideal experimental platform to study the impact of FPs on the behavior of their fusion partner.

INTRODUCTION:

Since the initial characterization of the green fluorescent protein (GFP) from *Aequorea victoria*[1], a wide palette of genetically encoded FPs have been developed, allowing cell biologists to simultaneously localize and track multiple cellular events/proteins in living cells[2,3]. FPs are derived from multiple organisms, from jellyfish to coral, and therefore, display specific

biophysical properties that divert extensively beyond their respective fluorescent spectrum. These properties include brightness, photostability, and a tendency to oligomerize among others[2,4]. Selecting monomeric FPs is an important aspect in the selection of a suitable tag when designing a fluorescent reporter, in order to minimize inappropriate interactions and alterations of the fusion partner's function and maximize the reporter efficiency for a given cellular compartment[4-6]. While GFP has, over time, been evolved to minimize the effect of adding the fluorescent tag to the fusion partner[5,7,8], how new FP variants perform compared to GFP remains difficult to assess.

Few methods exist to characterize the behavior of FPs. Most of them involve testing biophysical properties of FPs using biochemical approaches, such as ultracentrifugation and gel filtration protocols[9-12]. Such methods have the caveat of using purified FPs in solution, offering little insight into their behavior in intact cells. The development of the organized smooth endoplasmic reticulum (OSER) assay offers a quantifiable assessment of FPs' tendency to oligomerize in living cells[13] by testing the ability of overexpressed FPs to reorganize endoplasmic reticulum tubules into OSER whorls[14]. This technique can successfully detect changes between monomeric and oligomeric variants of GFP and other FPs. However, it relies mostly on overexpression in transiently transfected cells, and the quantitation and image analysis can be time-consuming unless the technique is adopted as an automated data collection and analysis workflow.

In order to complement these approaches, we established an assay that takes advantage of the effect of fluorescent tags on the toxicity and aggregation of polyQ expansions in yeast[15,16]. The expansion of the polyQ stretch with more than 36 repeats within the first exon of the gene encoding the huntingtin protein (Htt) is associated with Huntington's disease[17,18]. The expression of expanded Htt^{ex1} in yeast results in a strong aggregation of the misfolded Htt protein coupled to a severe growth defect. Interestingly, these phenotypes are strongly influenced by the sequences flanking the polyQ stretch, including FPs[15,16]. It was rationalized that the different properties of FPs can differentially affect polyQ toxicity in yeast. Indeed, compared to GFP-like FPs, red fluorescent proteins and their evolved forms have shown a reduced toxicity and aggregation[16]. This manuscript provides a detailed protocol to assess the effect of the next generation of FPs on polyQ toxicity and aggregation in yeast. This assay allows for a rapid and potentially high-content analysis of FP variants that can be used in parallel with previously characterized techniques for the optimal characterization of new FPs and can assess how they perform compared to GFP.

PROTOCOL:

1. Generation of New Fluorescently Tagged Htt^{ex1} Reporters for an Expression in Yeast

Note: This section has been modified from the protocol by Jiang *et al.*[16] and Albakri *et al.*[19].

1.1. Design primers to amplify the sequence encoding the fluorescent protein or interest by PCR. The forward primer should include a leader sequence to assist the restriction enzyme during digestion (GATC), followed by a SpeI restriction site (ACTAGT) and 20 bases downstream of the

ATG (excluding ATG) of the fluorescent protein gene of interest. The reverse primer should include the leader sequence (GATC), followed by a SalI restriction site (GTCGAC) and the reverse complement of 20 bases upstream of the stop codon of the FP sequence (including stop).

1.2. Using the primers designed in step 1.1, perform the PCR reaction using a thermocycler with the following settings: heat to 95 °C for 1 min and cycle at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min per kB of PCR product. Performing 18 cycles is ample.

1.3. Run the PCR reaction on an agarose gel (0.5% in Tris-acetate-EDTA). There should a single band corresponding to the expected product size. Isolate the fragment using a gel purification kit.

1.4. The protocol employs an Htt^{ex1} vector carrying 25 (nontoxic) and 72 (HD-associated, displaying a strong aggregation) polyQ repeats. Digest both the PCR fragments and the vector with SpeI and SalI restriction enzymes for 3 h at 37 °C.

1.5. Purify the digested vector by running it on an agarose gel as in step 1.3.

1.6. Purify the digested PCR fragment using a PCR purification kit.

1.7. Ligate the resulting digested PCR fragment and p415-*GAL1-FLAG*-25/72QpolyQ plasmids[1] using T4 ligase (1 h at room temperature). Use a 10 µL reaction (1 µL of T4 enzyme, 1 µL of 10X buffer, 6 µL of PCR fragment, and 2 µL of vector).

1.8. Transform 2 µL of the ligation reaction into 50 µL of *Escherichia coli*-competent cells and incubate them on ice for 30 min. Then, heat-shock the cells at 42 °C for 30 s. Add 1 mL of SOC outgrowth media and incubate at 37 °C for 1 h in a shaker. Plate 200 µL of the reaction on an LB-agar plate containing 100 µg/mL ampicillin. Incubate the plate at 37 °C overnight.

1.9. Select three individual bacterial colonies, grow them overnight in 3 mL of LB-broth containing 100 µg/mL ampicillin at 37 °C in a shaker and extract the plasmid DNA using a plasmid purification kit.

1.10. Check the plasmid by digesting 500 ng of DNA using SpeI and SalI restriction enzymes for 1 h at 37 °C and run the reaction on an agarose gel (0.5% in Tris-acetate-EDTA). There should be two bands at the right sizes of the vector (~7 kb) and the insert (size varies according to the gene of interest). Then, verify the plasmid by sequencing.

1.11. Transform the p415-*GAL1-FLAG*-polyQ-FP plasmids into the yeast strain W303 following a standard yeast transformation protocol[2].

2. Spotting Assay

2.1. Streak the yeast clones carrying 25Q/72Q tagged with the FP of interest on an agar plate

containing yeast selection media (synthetic complete-SC without leucine) with glucose as the carbon source. At the same time, also streak 25Q/72Q-ymsfGFP to serve as a positive control.

Note: 25Q/72Q constructs that do not contain a fluorescent tag are not toxic and can serve as negative control.

2.2. Incubate the plates at 30 °C for 2 - 3 d.

2.3. Select up to three single colonies from the plate.

2.4. Inoculate 5 mL of SC supplemented with 2% glucose as the carbon source.

2.5. Pellet 200 µL of each overnight culture and wash it 3x with sterile distilled water.

2.6. Resuspend the cells in SC media containing 2% galactose as the carbon source to induce the expression of polyQ fusions. Incubate the galactose media overnight at 30 °C in a tube rotator. As a control, repeat this step by using glucose-containing media.

2.7. The next morning, equalize the cell densities to optical density at 600 nm (OD_{600}) of 0.2 in 100 µL of SC media in a sterile 96-well plate.

2.8. Prepare four fivefold dilutions of each sample with sterile water by pipetting 20 µL of the sample from the previous well into 80 µL of media in the next well.

2.9. Use a yeast pinning tool to spot the cells onto selective plates (containing glucose or galactose) and incubate at 30 °C for 2 d.

2.10. Image the plates with an image documentation device.

3. Quantification of Cell Growth in Liquid Culture

3.1. Prepare the cell cultures, following steps 2.1 - 2.5 of this protocol.

3.2. Measure the OD_{600} using a spectrophotometer.

3.3. Dilute the cells to an OD_{600} of 0.1 in 300 µL of media in a 96-well plate.

3.4. Run each sample in triplicate.

3.5. Incubate the plate in a plate reader/incubator with shaking capabilities. Set the number of samples, the temperature at 30 °C, the absorbance at 600 nm, the length of the experiments to 24 h, and the measurement intervals to 15 min, and select the continuous shaking mode.

3.6. Create the growth curve and quantify the area under the curve using scientific graphing

software. The GraphPad Prism 7 is recommended. Paste the data into an XY table with three replicate values. The growth curve will be shown under the **Graphs** folder at the left side. To quantify the area under the curve, select **Analyze** at the top left and click **Area under curve** in **XY analyses**.

4. Fluorescent Microscopy

4.1. Prepare the cell cultures, following steps 2.1 - 2.5 of this protocol.

4.2. Dilute the cells 10x in growth media and transfer 200 µL of each sample to 8-well imaging chambers.

4.3. Image the cells using a confocal microscope equipped with a 63X Plan Aprochromoat objective (1.4 NA) at room temperature.

Note: The usage of a confocal microscope is optional. A standard wide-field fluorescent microscope can also be employed.

4.4. Adjust the pinhole and laser power for optimal image acquisition. Since the 72Q aggregates are much brighter than the diffuse 25Q signal, it is often required to use a different acquisition setting between the different plasmids in order to avoid saturation of the fluorescent signal.

4.5. Process the images using ImageJ[20] or another image-processing software. At this step, the percentage of cells that display aggregate can be calculated manually is desired.

5. Dot Blot

Note: In this protocol, dot blot is used to examine the protein expression levels. Prepare the cell cultures, following steps 2.1 - 2.5 of this protocol.

5.1. Generate protein lysates using glass beads in lysis buffer (100 mM Tris, pH 7.5; 200 mM NaCl; 1 mM EDTA; 5% glycerol, 1 mM dithiothreitol [DTT]). Add protease inhibitors, 4 mM phenylmethylsulfonyl fluoride (PSMF) and protease inhibitor cocktail, directly before use. Pellet 5 mL of the overnight culture and resuspend it in 200 µL of glass beads and 200 µL of lysis buffer. Vortex 30 s for 12 rounds. Centrifuge at 12,000 x *g* at 4 °C for 10 min and collect the supernatant.

5.2. Spot an equal amount of total proteins on a nitrocellulose membrane using a microfiltration apparatus. Prewet the membrane with PBS and assemble the apparatus. Connect to a vacuum source and make sure the screws are tightened. Turn on the vacuum and let the sample filter through the membrane by gravity.

5.3. Block the membrane in PBS- 0.05% Tween/5% fat-free milk.

5.4. Incubate the membrane with primary anti-FLAG antibody overnight at 4 °C. The monoclonal

anti-FLAG M1 is recommended.

5.5. Wash the membrane 3x for 10 min each with PSB- 0.05% Tween.

5.6. Incubate the membrane with a fluorescently labeled secondary antibody (anti-mouse IgG) for 1 h at room temperature in PBS- 0.05% Tween/5% fat-free milk.

5.7. Wash membrane 3x 10 min with PSB- 0.05% Tween.

5.8. Image-blot using an immunoblot documentation system.

REPRESENTATIVE RESULTS:

FPs have different biophysical properties, including their tendency to oligomerize, that can affect the behavior of their fusion partners in the context of fluorescent reporters. This protocol describes a simple method where multiple FPs can be fused to toxic polyQ expansions. Since polyQ toxicity is highly dependent on the sequences flanking the polyQ stretch[15], this assay allows a rapid and direct comparison of fluorescent polyQ fusion reporters (**Figure 1**). A non-HD-associated polyQ length (25Q) is used as a negative control and does not display significant toxicity or aggregation[15,16,21,22]. 72Q is employed to obtain the HD-like phenotypes, including strong growth inhibition and polyQ aggregation. Importantly, the Htt^{ex1} coding sequence employed lack the proline-rich domain that follows the polyQ stretch. In the presence of the proline-rich domain, Htt^{ex1} is not toxic in yeast[15]. In this assay, an Htt^{ex1} fused to a yeast-optimized monomeric variant of superfolder GFP[12] (ymsfGFP)[16] is used as a positive control as previously described[16]. The constructs also contain a FLAG epitope tag at the N-terminus of Htt^{ex1}. This allows detection of the different fusions with the same antibody (anti-FLAG) for biochemical analysis. As a proof-of-principle, 72Q Htt^{ex1} fused to yeast-optimized TagBFP (yomTagBFP)[23] does not result in slow growth measured by either spot assays on agar plates or growth in liquid media (**Figure 2**), indicating that the nature of the fluorescent tag can indeed impede polyQ expansion behavior in cells.

Aggregation of the fluorescent polyQ fusions can be assessed using fluorescent microscopy. 72Q-ymsGFP displays significant aggregation compared to 25Q. However, the 72-yomTagBFP fluorescent signal remains diffused throughout the cytoplasm (**Figure 3**). In most of the cases, it is not recommended to use the same image acquisition settings (laser power, exposure time) to acquire both 25Q and 72Q images. The aggregates in the 72Q-expressing cells are much brighter than the diffused 25Q signal. Therefore, under imaging conditions used to acquire 72Q images, the diffused 25Q signal may appear very weak or not be visible at all. Appropriate acquisition settings should also be applied to minimize the saturation during the imaging of the 72Q-expressing cells.

Expression levels of the various polyQ fusions could affect toxicity. Detergent-insoluble amyloids, such as polyQ aggregates, are notoriously difficult to study biochemically and are not suitable for an analysis by standerd SDS-PAGE. Therefore, dot blots can be performed to assess protein levels. The inclusion of the FLAG tag at the amino terminus end of Htt^{ex1} allows detection of all the

fluorescent fusions simultaneously, despite the presence of FPs (**Figure 4**). Alternatively, semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) can be performed to assess the formation of polyQ oligomers[16]. A detailed protocol and video are available in Halfmann and Lindquist[24].

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow diagram for the analysis of the effect of fluorescent protein tag on the aggregation and toxicity of polyQ expansion proteins in yeast. First, FPs are cloned into yeast expression vectors encoding a galactose-inducible version of FLAG-tagged Htt^{ex1} harboring either 25Q (nontoxic) or 72Q (HD-associated, aggregating and toxic) repeats. Clones are selected and verified by sequencing and, subsequently, transformed in yeast. Following the induction of polyQ fusion expression by incubation in galactose-containing media, either spotting assays on agar plates or growth liquid media can assess the polyQ toxicity. PolyQ aggregation is analyzed by fluorescent microscopy. A relative expression of the different constructs is assessed using dot blot.

Figure 2: Representative growth assay results following the expression of Htt^{ex1} fluorescent fusions in yeast. Yeast expressing either 25Q or 72Q Htt^{ex1} fused to ymsfGFP or yomTagBFP was cultured in glucose (control) or galactose media (polyQ-induced) overnight and either (**A**) spotted on agar plates or (**B**) incubated further in liquid media to assess growth under the different conditions. While 72Q-ymsfGFP induces a significant growth defect, 72Q-yomTagBFP displays a growth phenotype similar to the nontoxic 25Q counterparts.

Figure 3: Representative fluorescent images of Htt^{ex1} fluorescent fusions in yeast. Yeast expressing either 25Q or 72Q Htt^{ex1} fused to ymsfGFP or yomTagBFP was cultured in glucose (control) or galactose media (polyQ-induced) overnight and imaged with a confocal microscope. While the 72Q-ymsfGFP expression results in a strong polyQ protein aggregation, 72Q-yomTagBFP displays a diffused cytoplasmic signal similar to the nontoxic 25Q counterparts.

Figure 4: Representative dot blot analysis of Htt^{ex1} fluorescent fusion expression in yeast. Yeast expressing 25Q, 46Q, 72Q, or 103Q Htt^{ex1} fused to CFP was cultured in galactose media (polyQ-induced) overnight and processed for dot blot analysis. Fivefold dilutions of the cell lysates are shown.

DISCUSSION:

In this article, various assays to measure the aggregation of Htt^{ex1} polyQ expansions and their effect on yeast growth were employed as a model to study how different fluorescent proteins alter their fusion partners in the context of fluorescent reporters. Using a GFP variant (ymsfGFP) as a positive control, we showed that this detects significant changes in polyQ toxicity and aggregation between different fluorescent tags and allows for a direct and rapid comparison of the polyQ-FP fusion performance against GFP-tagged constructs[16,19].

While the present protocol focuses on fluorescent proteins, various parts of the protocol could

be readily adapted to test the effects of other protein tags. In addition, the present protocol employs low-copy yeast centromeric vectors that can vary in terms of copy numbers (generally one to two copies) present in cells[25]. Using integrative vectors to ensure a uniform expression across experimental conditions could circumvent this problem. While this protocol has been optimized for use in the W303 background, other *S. cerevisiae* strains can be employed. However, susceptibility to polyQ toxicity should be determined using the ymsfGFP-tagged vectors prior to designing new constructs. In certain cases, it may be appropriate to employ high-copy (2µ) vectors to generate a significant growth defect. It is also suggested to test multiple isolates following the yeast transformation with polyQ vectors to avoid selecting spontaneous suppressors showing a reduced polyQ toxicity. Of note, the W303 yeast strain[26] is usually used as it is more sensitive to polyQ toxicity than other S288C derivatives, such as BY4741/BY4742[27], thus allowing for a wider range of growth phenotypes. Importantly, strains employed for this assay need to carry the Rnq1 prion protein since *rnq1*Δ cells do not display polyQ toxicity and aggregation[28]. It is also important to use Htt^{ex1} constructs carrying the amino-terminal FLAG tag and lacking the proline-rich domain. Other variations of the fusion design may alter toxic phenotypes[15]. Finally, the induction of the polyQ fusion expression in galactose-containing media is a critical step of the protocol[21]. When transferring cells from glucose- to galactose-containing media, it is important to wash the cells at least three times with sterile water to eliminate all traces of glucose that could contribute to repressing the induction of the Gal1 promoter[29]. When performing spotting assays, culturing the cells overnight in galactose media to induce the expression of the fusion can exacerbate the toxic phenotype of the 72Q fusion and help discriminate changes in growth across different fusions[16].

As a limitation, previous studies did not observe differential effects between a nonmonomeric version of CFP (a GFP derivative) and ymsfGFP[16]. Thus, at least for GFP variants, this assay may not be sensitive enough to discriminate between monomeric and oligomeric variants, highlighting the need to complement the polyQ toxicity assays with other standard methods, such as the OSER assay[13] and biochemical analysis[9-12] that can directly assess oligomerization. Also, it should be noted that FPs can behave differently in yeast compared to *in vitro* assays or their expression in other organisms[23].

Collectively, these methods allow researchers to rapidly characterize new FPs and measure their effect on their fusion partner. In the future, this protocol will enable the quick screening of new derivatives of previously characterized FPs to identify mutants that behave similarly to GFP variants, which are still the gold standard measure for FP reporters. While this protocol focuses on fluorescent proteins, it can easily be adapted to screen for the effects of other genetically encoded tags, such as SNAP-tag[30] and SunTag[31].

In conclusion, this protocol provides a rapid and easily scalable assay to enable further characterization of the new generation of FPs and other genetically encoded tags to guide research in fusion protein design.

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DISCLOSURES:

The authors have nothing to disclose.

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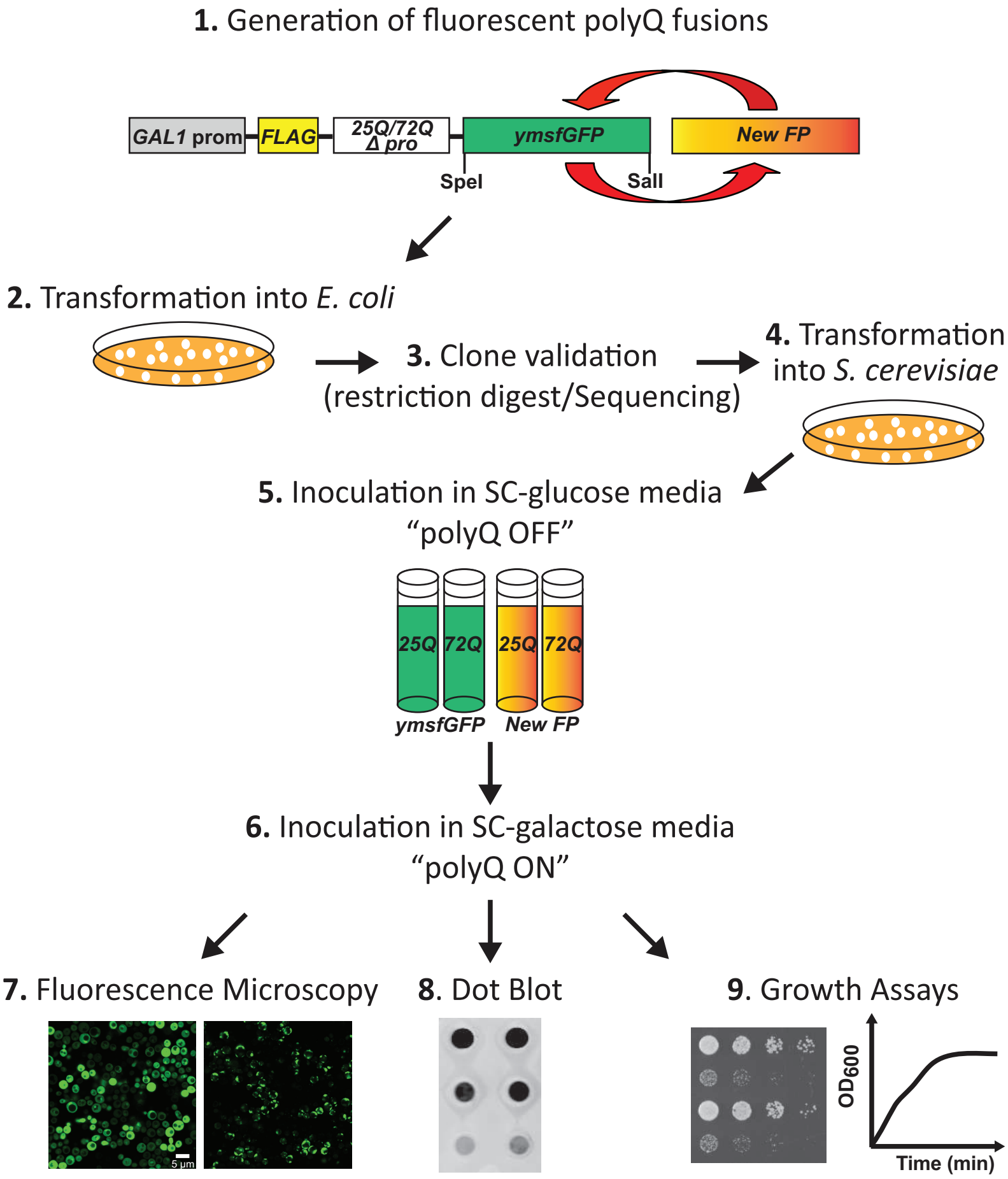

Figure 1

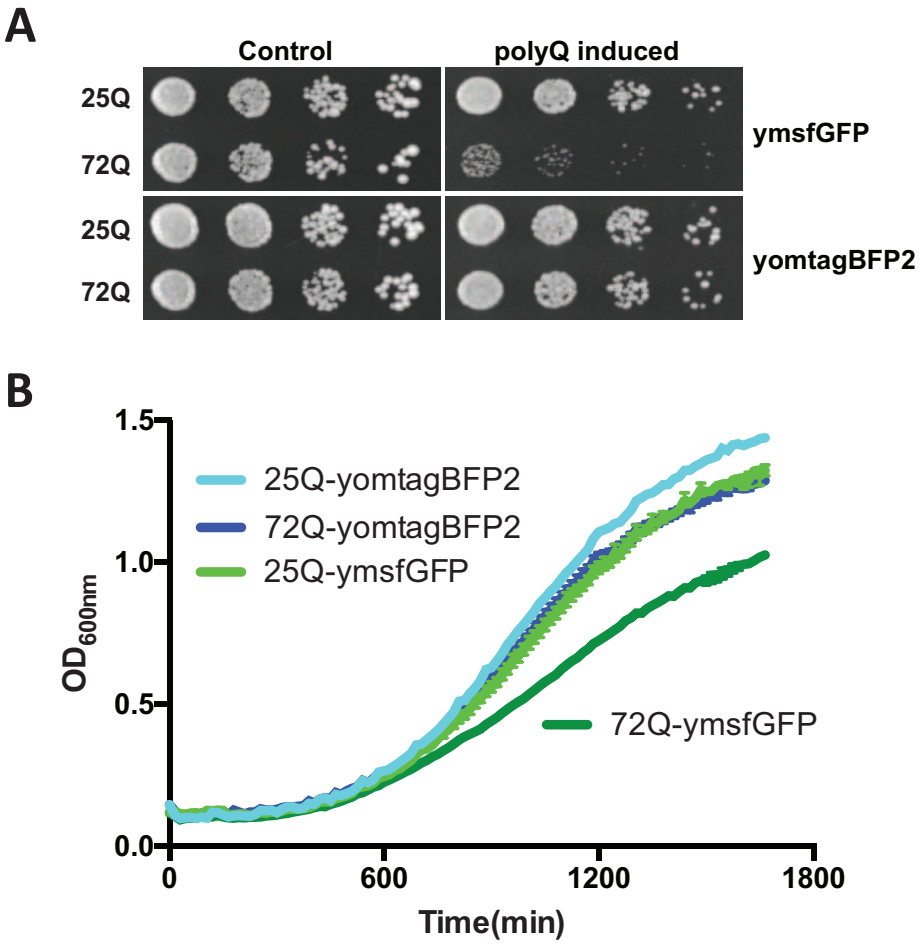

Figure 2

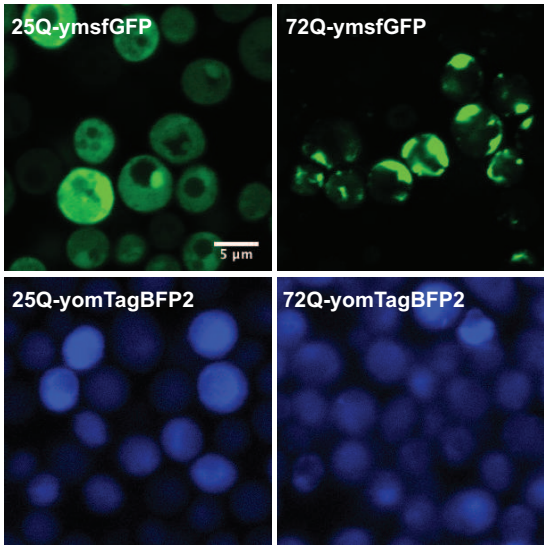

Figure 3

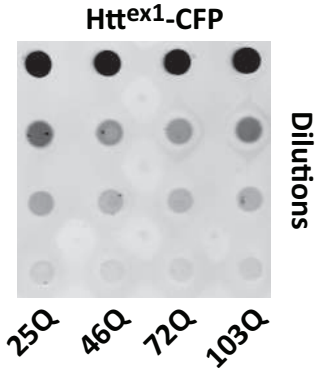

Figure 4

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
5-alpha Competent E. coli (High efficiency)	New Englanfd Biolab	C2987	
SpeI-HF	New Englanfd Biolab	R3133	High fidelity enzymes are preferre
SalI-HF	New Englanfd Biolab	R0138	High fidelity enzymes are preferre
Agarose	Fisher Scientific	BP160	
LB-Agar	Fisher Scientific	BP1425	
LB-Broth	Fisher Scientific	BP1426	
Ampicilin	Fisher Scientific	BP1760	
PfuUltra High-fidelity DNA Polymerase	Agilent Technologies	600382	
EPOCH2 microplate spectrophotometer	BioTek Instruments inc	EPOCH2TC	
Yeast Pin Replicator	V&P Scientific inc.	VP407AH	
SPI imager	S&P Robotics inc.	spImager-M	
Zeiss LSM 800 confocal with AryScan	Carl Zeiss Microscopy	LSM 800	
8 well Lab-Tek imaging chambers	Fisher Scientific	12565470	
Bio-Dot apparatus	Bio-Rad	1706545	
Chemi Doc XRS+	Bio-Rad	1708265	
anti-FLAG M1 antibody	Sigma-Aldrich	F3040	
Goat anti-mouse IgG alexa 555 secondary antibody	Thermo	A32727	
Plasmid MiniPrep Kit	Fisher Scientific	K0503	
Plasmid Gel extraction Kit	Fisher Scientific	K0831	
PCR Purification Kit	Fisher Scientific	K0702	
Prizm	GraphPad	N/A	
TAE (Tris-Acetate-EDTA)	Fisher Scientific	BP13354	

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Title of Article: Assessing the effects of fluorescent proteins on fusion partners using polyQ toxicity assays in yeast

Author(s): Jiang, DiGregorio, Alkaki, Duennwald, Lajoie

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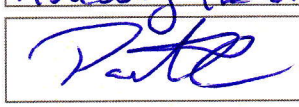
Signature: 

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Western University
London, Ontario, CANADA

August 30th 2018,

Editor *JOVE*

Dear Editor,

We are pleased to resubmit our manuscript titled “Assessing the effect of fluorescent proteins on fusion partners using polyglutamine toxicity assays in yeast -58748_R1” by Yuwei Jiang, *et al*. for consideration as a video protocol in *JOVE*.

We believe that we have addressed all the reviewers’ comments and that our manuscript is now acceptable for publication in *JOVE*.

Best regards,

Patrick Lajoie, Ph.D.

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proof read the manuscript.

2. Please specify the primers for PCR reactions.
The primers would be the one generated in step 1.1. We have specified so in the text.

3. Figure 1. Please add a scale bar to the Fluorescence Spectra.
Done.

4. Figure 2A: Please add scale bars.
There is no fluorescent micrograph in this figure.

5. Step 1.1: Please specify how to design the primers. Please add more details. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.
We have added details about the composition of the different primers. No software is required.

6. 1.9: Please specify the condition for growing the colonies.
We have included the temperature.

7. 3.1: If you want to film this step, Step 2.1-2.5 must be highlighted.
We have highlighted steps 2.1-2.5.

8. 3.6: How to create? What software is used?
We now mention the software and how to use it.

9. Please specify the antibodies used in the protocol.
We now mention explicitly the anti-FLAG and appropriate secondary antibody.

10. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.
Done.

11. Please use a single space between numerical values and their units.
Done.

12. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
Done.

13. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
We now mention critical steps in the protocol, namely the induction in galactose media and the need to use a RNQ1+ strain.

b) Any modifications and troubleshooting of the technique
We mention that the assay can be adapted to other yeast strains but that it may require additional modifications to the protocols that are discussed.

c) Any limitations of the technique

Limitations are acknowledged. Mainly the inability of the assays to discriminate between oligomeric and monomeric species of GFP variants and the fact that FPs can shows different behavior in yeast compared to other organisms.

d) The significance with respect to existing methods
We now mention that the assay has the advantage of being easily adaptable to high content screens compared to other methods.

e) Any future applications of the technique

We mention that the assay can be used to test the impact of the next generation of FPs, or to screen mutants of existing FPs. In addition, it could be employed to test the effects of other tags, such as SNAP tag.