



RESEARCH NOTE

REVISED Polyglutamine toxicity assays highlight the advantages of mScarlet for imaging in *Saccharomyces cerevisiae* [version 2; referees: 2 approved]

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Abstract

Development of fluorescent proteins (FPs) enabled researchers to visualize protein localization and trafficking in living cells and organisms. The extended palette of available FPs allows simultaneous detection of multiple fluorescent fusion proteins. Importantly, FPs are originally derived from different organisms from jelly fish to corals and each FP displays its own biophysical properties. Among these properties, the tendency of FPs to oligomerize inherently affects the behavior of its fusion partner. Here we employed the budding yeast *Saccharomyces cerevisiae* to determine the impact of the latest generation of red FPs on their binding partner. We used a yeast assay based on the aggregation and toxicity of misfolded polyQ expansion proteins linked to Huntington’s disease. Since polyQ aggregation and toxicity are highly dependent on the sequences flanking the polyQ region, polyQ expansions provide an ideal tool to assess the impact of FPs on their fusion partners. We found that unlike what is observed for green FP variants, yemRFP and yFusionRed-tagged polyQ expansions show reduced toxicity. However, polyQ expansions tagged with the bright synthetically engineered ymScarlet displayed severe polyQ toxicity. Our data indicate that ymScarlet might have significant advantages over the previous generation of red FPs for use in fluorescent fusions in yeast.

Keywords

fluorescent proteins, mScarlet, yeast, polyglutamine toxicity, aggregation, Huntington’s disease

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Genereaux J: Data Curation, Formal Analysis; **Lajoie P:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED Amendments from Version 1

In this revised version, we added quantitative growth analysis in liquid cultures to compare toxicity of the different fluorescent fusions. We also added quantification for the dot blot experiments. Finally, to determine if the absence of toxicity of the yFusionRed-tagged 68Q construct was due to its lower expression, we expressed the fusion from a multicopy vector. This resulted in Htt^{ex1}-yFusionRed toxicity and aggregation, albeit to a lower extent compared to the ymsfGFP-tagged counterpart.

In [Figure 1](#) we have changed the statistical significance of the data in [Figure 1D](#). In [Figure 2](#) we added growth curves to 2B and have removed the dot blot which is now [Figure 3](#). We generate a new [Figure 4](#) in response to reviewer comments. Julie Genereaux, who generated the plasmids used in [Figure 4](#), has been added as a contributing author. Additional raw data behind [Figure 1–Figure 4](#) has been added to [Dataset 1](#).

See referee reports

Introduction

Following the development of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* ([Chalfie et al., 1994](#)), several other FPs with various spectral properties have been characterized ([Thorn, 2017](#)), allowing simultaneous detection of multiple fluorescent reporters. Among the most popular alternatives to GFP are the red fluorescent proteins (RFPs) isolated from *Anthozoa* coral and anemone species. One of the drawbacks of RFPs is that *Anthozoa* derived FPs are obligate tetramers ([Baird et al., 2000](#); [Verkhusha & Lukyanov, 2004](#)). While development of RFPs into monomeric versions has been successful, it is often associated with reduced brightness of the fluorescent signal ([Campbell et al., 2002](#)) and therefore reduced overall performance of the resulting monomeric FPs. Moreover, RFPs such as TagRFP and mRuby2 reported as monomeric by passing purified proteins through sizing columns still display high tendency to oligomerize in living mammalian cells ([Costantini et al., 2015](#); [Costantini et al., 2012](#)). Thus, under specific circumstances, FPs reported as monomeric can still be prone to oligomerization. Unwanted formation of oligomers could potentially significantly alter the function/localization of the protein of interest fused to the FP and render reporters unreliable ([Costantini et al., 2015](#); [Snapp et al., 2003](#); [Zacharias et al., 2002](#)). Indeed, various RFPs (mCherry, mKate2, mRuby, mKO2, mApple, TagRFP-T) have been shown to have differential effects on localization of *cdc12* in yeast ([Lee et al., 2013](#)). Thus, being able to assess the behavior of fluorescent reporter in a given organism and/or cellular compartment is critical to help optimize fluorescent reporter design ([Snapp, 2009](#)).

We recently established a method to rapidly compare the behavior of FPs against a monomeric variant of superfolder GFP (msfGFP) in yeast ([Jiang et al., 2017](#)). The assays exploit the ability of polyglutamine expansions associated with Huntington's disease (HD) to form toxic aggregates in yeast cells. The cause of HD can be traced back to abnormal expansion of a polyQ stretch within the first exon of the gene encoding the Huntingtin

protein (Htt^{ex1}) resulting in chorea and cognitive defects in patients ([Gusella & MacDonald, 1995](#); [Huntington, 1872](#); [Penney et al., 1997](#)). Expansion over 36 repeats is known to cause the Htt protein to misfold and aberrantly accumulate into detergent-insoluble amyloid-like aggregates in the cytoplasm of striatal neurons ([Penney et al., 1997](#)). Expression of expanded Htt^{ex1} in yeast results in severe polyQ aggregation and growth defect ([Duennwald, 2013](#); [Krobitsch & Lindquist, 2000](#); [Mason & Giorgini, 2011](#); [Meriin et al., 2002](#)). Interestingly, the nature of the sequences flanking the polyQ regions (in this case fluorescent or epitope tags) greatly affects the propensity of the polyQ expansions to aggregate and to display significant growth defects in yeast ([Duennwald et al., 2006](#)). Using polyQ toxicity assays in yeast, we previously showed that a yeast-optimized version of mCherry (termed yemRFP ([Keppler-Ross et al., 2008](#))) displays only a mild growth defects compared to yeast-optimized msfGFP (ymsfGFP) ([Jiang et al., 2017](#)). These results lead us to exploit the polyQ toxicity and aggregation assays to explore the effects of two of the most recently available RFPs. Here, we focused on FusionRed, a red monomeric fluorescent variant of mKate2 known for its low cytotoxicity in cells ([Shemiakina et al., 2012](#)) that displays low propensity to oligomerize in mammalian cells ([Costantini et al., 2015](#)). We also included mScarlet, a monomeric synthetic RFP that was recently shown to outperform other RFPs in terms of brightness of the fluorescent signal ([Bindels et al., 2017](#)). Both have yet to be characterized for expression in yeast.

Methods

Yeast strains and culture conditions

All strains are derived from W303-1A ([Thomas & Rothstein, 1989](#)). All experiments were conducted in synthetic complete media (SC) at 30°C.

DNA constructs

yemRFP ([Keppler-Ross et al., 2008](#)) was previously described. yFusionRed and ymScarlet were codon optimized for expression in yeast and synthesized by Genscript Inc. based on previously published sequences ([Bindels et al., 2017](#); [Shemiakina et al., 2012](#)). RFPs were cloned into the SpeI/SalI site of p415 GPD. Alternatively, RFPs were cloned into the SpeI/SalI sites of p415 GAL1 25Q/68Q Htt^{ex1} lacking the proline rich domain, as previously described ([Jiang et al., 2017](#)). To generate 2μ vectors, the GAL1 25Q/68Q Htt^{ex1}-ymsfGFP or GAL1 25Q/68Q Htt^{ex1}-yFusionRed fragments were cloned into the SacI/SalI sites of pRS42N ([Taxis & Knop, 2006](#)). All Htt^{ex1} constructs lack the proline-rich domain since absence of this domain is required for Htt^{ex1} toxicity in yeast ([Duennwald et al., 2006](#)). We also noted that since the publication of our previous study ([Jiang et al., 2017](#)), the original 72Q Htt^{ex1} plasmid has mutated into 68Q. We, therefore, used the latter in this study. See [Table 1](#) for a list of plasmids used in this study.

Growth assays

Yeast growth was measured by spotting assay on agar plates. Briefly, cells were cultured overnight to saturation in appropriate

Table 1. Plasmids used in this study.

Plasmids	Resistance marker	Source
P415 GPD	Leu	(Mumberg <i>et al.</i> , 1995)
P415 GPD-yemRFP		This study
P415 GPD-yFusionRed		
P415 GPD-ymScarlet		
P415 Gal1-FLAG-25Q-ymsfGFP		
P415 Gal1-FLAG-68Q-ymsfGFP		This study
P415 Gal1-FLAG-25Q-yemRFP		
P415 Gal1-FLAG-68Q-yemRFP		This study
P415 Gal1-FLAG-25Q-yFusionRed		
P415 Gal1-FLAG-68Q-yFusionRed		
P415 Gal1-FLAG-25Q-ymScarlet		
P415 Gal1-FLAG-68Q-ymScarlet		
PRS42N Gal1-FLAG-25Q-ymsfGFP	natNT2	
PRS42N Gal1-FLAG-68Q-ymsfGFP		
PRS42N Gal1-FLAG-25Q-yFusionRed		
PRS42N Gal1-FLAG-68Q-yFusionRed		

selection media. The next day, cells densities were equalized to OD₆₀₀ 0.2 and 5x serial dilutions were spotted on agar plates. Alternatively, cell densities were equalized to OD₆₀₀ 0.1 and cells 300 μ L of cell suspensions were transferred into a 96 well plate and incubated at 30°C for 24h with constant shaking in a Biotek Epoch 2 microplate spectrophotometer and OD₆₀₀ was recorded every 15 minutes.

Dot blot

After induction in galactose media overnight, cells were lysed using glass beads in lysis buffer (100 mM Tris pH 7.5; 200 mM NaCl; 1 mM EDTA; 5% glycerol, 1 mM Dithiothreitol (DTT) 4 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail). Equal amount of proteins were spotted on a nitrocellulose membrane. Membranes were blocked for 30 min in PBS-0.05%Tween at room temperature were and then processed for immunoblot. Membranes were probed with anti-FLAG primary antibody (Sigma F3040, 1:5000 dilution) overnight at 4°C and subsequently with a secondary anti-mouse fluorescent antibody (Thermo Alexa 555 #A21424, 1:5000 dilution) for

1h at room temperature and imaged using a Bio-Rad ChemiDoc MP imaging system. Membranes were then stripped using the Gene Bio-Application stripping buffer and reprobbed with an anti-Pgk1 primary antibody (Thermo 22C5D8) using the same secondary antibody. In **Figure 3**, for each individual antibody, both membranes were imaged simultaneously to allow direct comparison of fluorescent signal. Densitometric analysis was performed using Image J.

Fluorescence microscopy

Under the different experimental conditions, cells were diluted 10x in growth media and plated in Lab-tek (Thermo Inc.) imaging chambers and processed for fluorescence microscopy. Images in **Figure 1** were acquired using a Zeiss AxioVert A1 wide field fluorescence microscope equipped with a 63X NA 1.4 Plan Apochromat objective, a 560 to 600nm excitation/630 to 705 nm emission bandpass filter and Zeiss AxioCam 506 mono camera. Images presented in **Figure 2** and **Figure 4** were collected using a Zeiss 800 confocal microscope equipped with 488 nm and 561 nm diode lasers and a 63x PlanApochromat NA 1.4 objective.

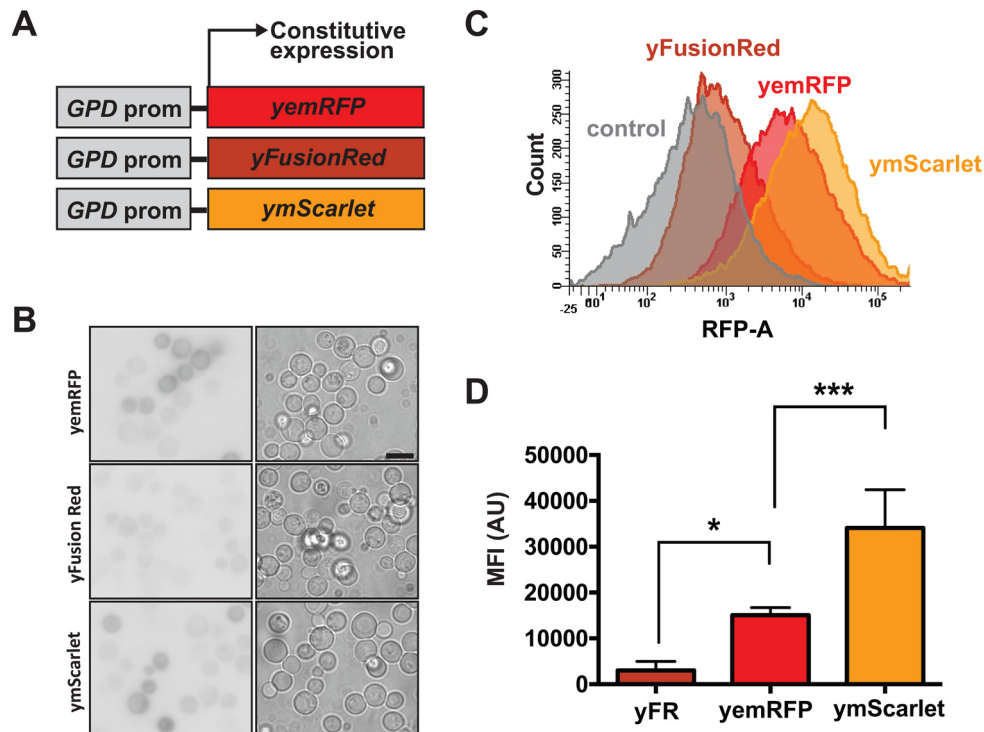


Figure 1. Comparison of red fluorescent proteins (RFPs) fluorescent intensities in yeast. (A) Scheme of fluorescent proteins vectors. yemRFP, yFusionRed and ymScarlet were introduced into centromeric vectors under the control of a constitutive GPD promoter. (B) Representative images from 3 fields of yeast cells expressing different RFPs. Imaging conditions were kept constant between samples to allow direct comparison of fluorescent intensities. Inverted black and white images are shown for clarity. Bar: 5µm (C) Yeast cells expressing the different RFPs were analyzed by flow cytometry and compared to cells carrying an empty vector. (D) Median fluorescent intensities of the various RFPs were calculated from fluorescent data acquired using flow cytometry. * $p < 0.05$ and *** $p < 0.001$ according to a one way ANOVA followed by a Dunnett's multiple comparison test comparing samples to yFusionRed.

Flow cytometry

Cells were cultured with appropriate media and processed for flow cytometry using a BD Bioscience FACS Celesta flow cytometer equipped with a 561 Yellow laser for imaging of RFPs. Data were analyzed using the BD FACS Diva software. All conditions were performed in triplicates, 20,000 cells were analyzed and median fluorescence intensities were calculated. No gates were applied.

Statistical analysis

A one way ANOVA followed by a multiple comparison test (Tukey's or Dunnett's according to figure legends) was used to determine statistical significance between the different experimental conditions in Figure 1D, Figure 2B, Figure 3 and Figure 4A using GraphPad Prism v6.0h.

Results and discussion

To analyze the performance of the three different RFPs in yeast, we first generated codon optimized versions of both FusionRed and mScarlet (termed yFusionRed and ymScarlet, respectively) (Table 2). Centromeric plasmids encoding

yFusionRed, yemRFP and ymScarlet under the control of the constitutive GPD promoter were transformed in yeast (Figure 1A). Fluorescence intensities were compared using wide-field fluorescence microscopy (Figure 1B). Median fluorescence intensity (MFI) was then quantified using flow cytometry. Quantification revealed that yFusionRed was significantly dimmer (~5x) than yemRFP (Figure 1C and D). This result was surprising given that previously published data reported a slightly increased brightness for FusionRed when compared to mCherry (Shemiakina *et al.*, 2012). However, it is known that fluorescent brightness of FPs expressed in yeast can be different from the ones registered for pure purified proteins (Lee *et al.*, 2013). As opposed to yFusionRed, ymScarlet displayed the strongest fluorescent signal (~2x brighter than yemRFP) (Figure 1C and D). These results are in agreement with previous studies reporting increased brightness of mScarlet compared to other RFPs variants (Bindels *et al.*, 2017). Based on the intensity of the fluorescent signal, ymScarlet appears to be the optimal RFP for imaging in yeast.

Dataset 1. Raw data behind Figures 1, 2, 3 and 4

<https://dx.doi.org/10.5256/f1000research.15829.d225385>

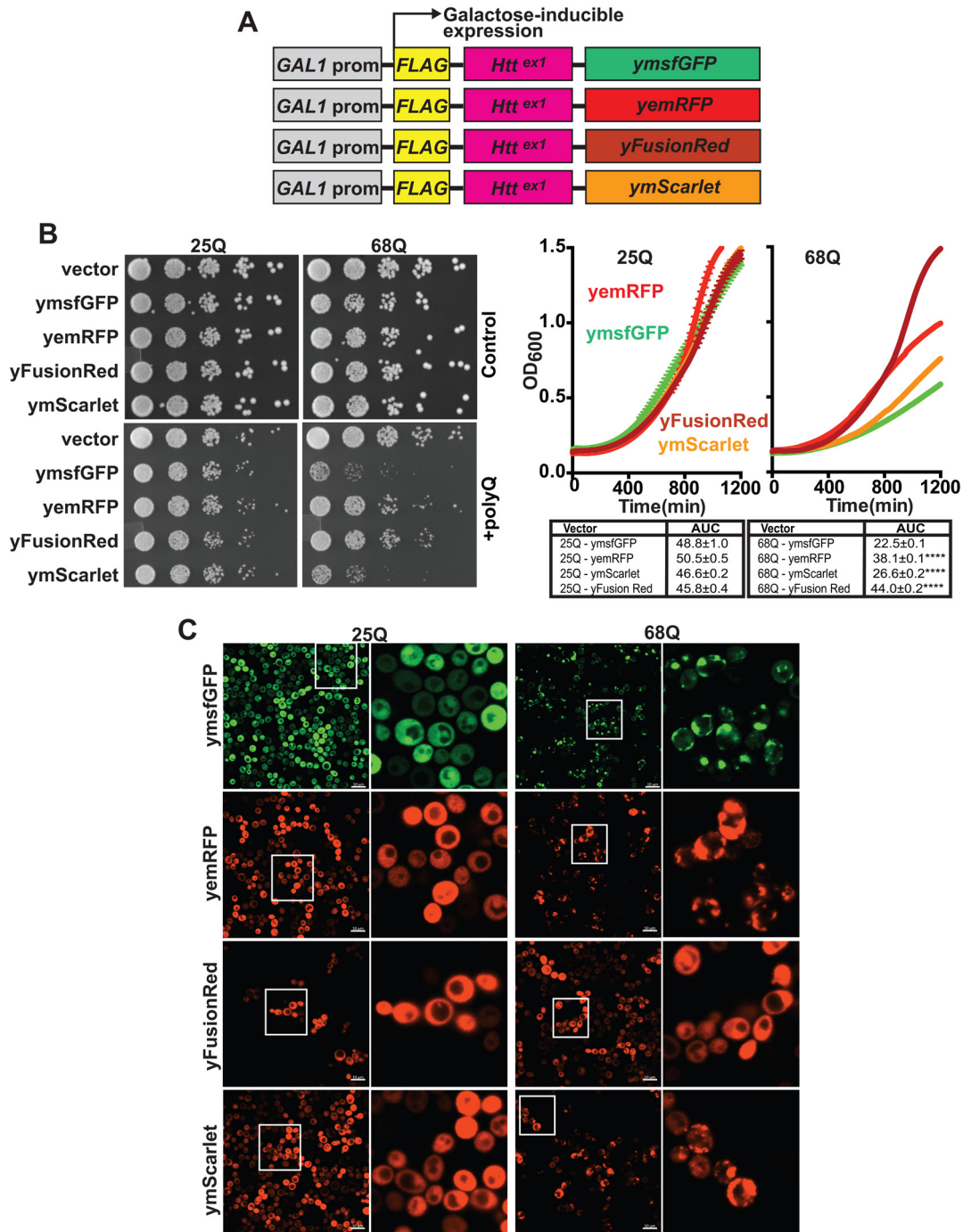


Figure 2. Unlike yFusionRed, ymScarlet displays a toxic polyQ phenotype similar to ymsfGFP. (A) Scheme of vectors encoding fluorescent proteins (FPs) were cloned in frame with FLAG-Htt^{ex1} into a centromeric vector carrying a *GAL1* inducible promoter. (B) Images of yeast growth assays on agar plates. Yeast cells carrying an empty vector or 25/68Q Htt^{ex1} fused to either ymsfGFP, yemRFP, yFusionRed or ymScarlet were grown to saturation overnight in glucose (control) or galactose (polyQ induced) containing media. The next days, cell concentrations were equalized to OD₆₀₀ 0.2 and 5 fold serial dilutions of the cell suspension spotted on synthetic complete agar media plates containing either glucose or galactose. Alternatively, cells were cultured in liquid media and optical densities were recorded over time to generate growth curves. The area under the curve (AUC) was calculated from 3 experimental replicates. ****p<0.0001 according to a one way ANOVA followed by a Dunnett's multiple comparison test comparing samples to ymsfGFP-tagged fusion of the same polyQ length. (C) Representative fluorescent images from 3 fields of yeast cells expressing 25/68Q Htt^{ex1} fused to ymsfGFP, yemRFP, yFusionRed or ymScarlet after overnight induction in galactose-containing media.

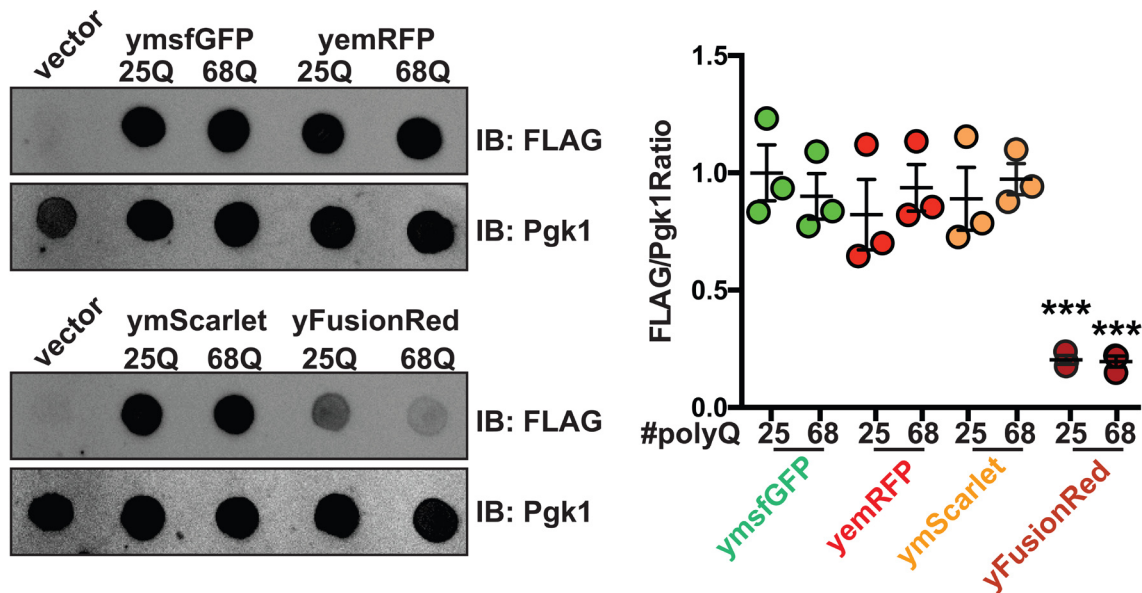


Figure 3. yFusionRed-tagged Htt^{ex1} fusions are expressed at lower levels compared to other fluorescent fusions. (A) Yeast cells carrying an empty vector or 25/68Q Htt^{ex1} fused to ymsfGFP, yemRFP, yFusionRed or ymScarlet or carrying an empty vector were induced overnight in galactose containing media and protein levels analyzed by dot blot using either an anti-FLAG (detection of fluorescent fusions) or anti-Pgk1 antibody (loading control). Quantification of the FLAG/Pgk1 ratio is shown from 3 independent experiments. *** $p < 0.001$ according to a one way ANOVA followed by a Tukey's multiple comparison test comparing samples to ymsfGFP-tagged fusion of the same polyQ length.

Next, we sought to determine how the three different FPs affect their fusion partners in living yeast. To this end, we employed the polyQ toxicity assays. Each RFP was cloned in frame with a galactose inducible version of Htt^{ex1} carrying either 25Q (non-pathological length) or 68Q (HD-associated) (Figure 2A). 25Q constructs show no growth differences across the different FPs in both uninduced (glucose media) and polyQ-induced (galactose media) conditions indicating that expression of the different constructs results in similar growth phenotypes. When fused to 68Q Htt^{ex1}, yFusionRed displayed no significant toxicity when compared to the non-toxic 25Q fusion (Figure 2B). Interestingly, ymScarlet displayed severe toxicity, showing a slow growth phenotype comparable to what was observed for ymsfGFP (Figure 2B). Based on these observations, we then investigated the effects of the different RFPs on polyQ aggregation using fluorescence microscopy. We found that yemRFP displayed robust 68Q aggregation similar to ymsfGFP as we previously described (Jiang *et al.*, 2017). It is important to note that while prone to aggregation, yemRFP polyQ proteins were shown to form aggregates with different biophysical properties (increased detergent solubility) that can account for their moderately toxic nature (Jiang *et al.*, 2017). In accordance with the absence of toxicity noted in the growth assay, 68Q-FusionRed did not form visible aggregates, while ymScarlet displayed strong aggregation

propensity (Figure 2C). In addition, assessment of protein abundance for each constructs using dot blot revealed that both 25 and 68Q yFusionRed fusions were present at lower levels compared to other fluorescent counterparts (Figure 3A). The cause of this phenotype is unclear and could result from increased turnover rate of the fusions. Interestingly, expression of 69Q-yFusionRed from a multicopy 2 μ vector resulted in a growth defect, albeit toxicity was reduced compared to ymsfGFP-tagged polyQ (Figure 4A). Moreover, under these conditions, 68Q-yFusionRed displayed robust aggregation. This indicates that the lower expression levels observed for yFusionRed constructs can potentially explain, at least partially, the absence of polyQ toxicity when expressed at lower levels. Reduced toxicity of the 68Q-yFusionRed is also consistent with our previous observation showing that yomTagBFP2, a blue fluorescent proteins similarly does not form toxic aggregates (Jiang *et al.*, 2017). In fact, both FusionRed and mTagBFP2 (Subach *et al.*, 2011) are evolved versions of the wild-type RFP from sea anemone *Entacmaea quadricolor* (Merzlyak *et al.*, 2007). In the case of mScarlet, the protein was evolved from a synthetic template design for generating a monomeric protein. Therefore, based on our data, mScarlet appears to be an attractive alternative to mCherry, which minimizes the effect of the FP on its fusion partner.

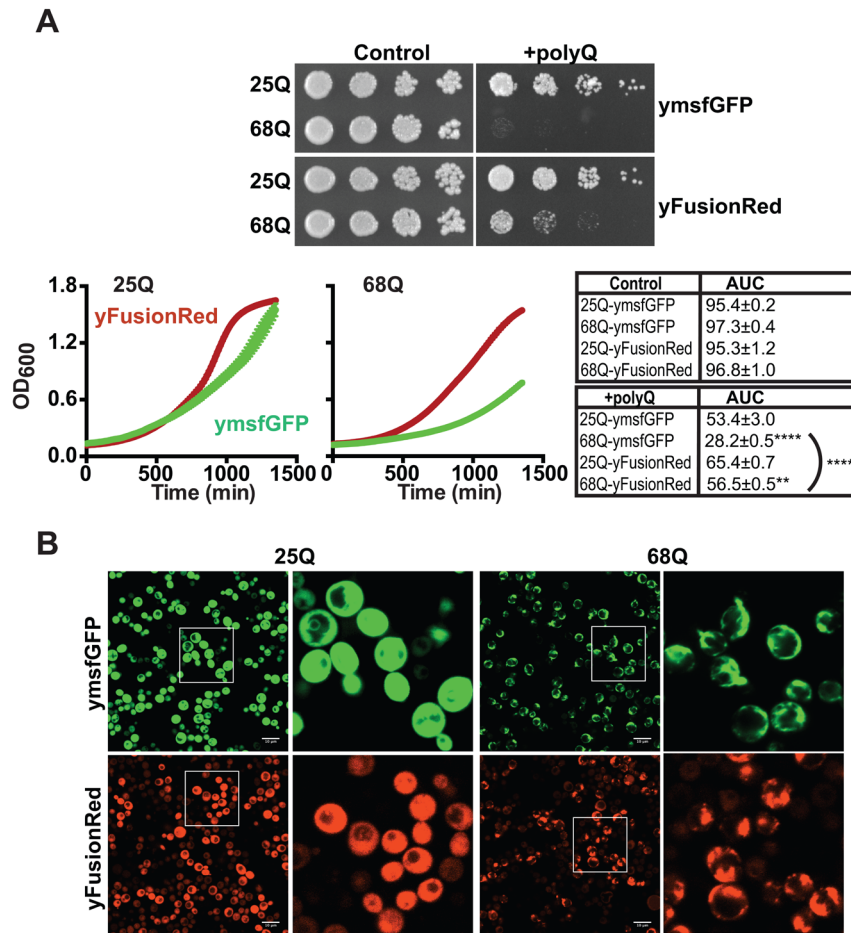


Figure 4. Expanded Htt^{ex1}-yFusionRed is toxic when expressed at high levels. (A) Images of yeast growth assays on agar plates. Yeast cells carrying 25/68Q Htt^{ex1} fused to either ymsfGFP or yFusionRed in a 2 μ multicopy vector were grown to saturation overnight in glucose (control). The next days, cell concentrations were equalized to OD₆₀₀ 0.2 and 5 fold serial dilutions of the cell suspension spotted on synthetic complete agar media plates containing either glucose (control) or galactose (+polyQ). Alternatively, cells were cultured in liquid media and optical densities were recorded over time to generate growth curves. The area under the curve (AUC) was calculated from 3 experimental replicates. **p<0.01 and ****p<0.0001 according to a one way ANOVA followed by a Tuckey's multiple comparison test comparing the 68Q sample to its 25Q counterpart unless indicated otherwise. **(B)** Representative fluorescent images from 3 fields of yeast cells expressing 25/68Q Htt Htt^{ex1} fused to ymsfGFP or yFusionRed from a multicopy 2 μ vector after overnight induction in galactose-containing media. Under these conditions, 68Q-yFusionRed displays robust aggregation.

Table 2. Sequences of yeast optimized fluorescent proteins generated in this study.

Name	Sequences
yFusionRed	ATGGTTTCTGAATTGATTAAAGAAAACATGCCAATGAAGTTGTACATGGAAGGTTACTGTTAACACCATCATTTTAAATGTACATC AGAAGGTGAAGGTAAACCATACGAAGGTACTCAACAATGAGAATTAAGTTGTTGAAGGTGGTCCATTGCCATTTGCTTTTCGA TATTTGGCAACTTCTTTATGTACGGTTCAAGAACTTTTATTAAGCATCCACCAGGTATTCCAGATTTCTTTAAGCAATCTTTCCCA GAAGGTTTTACTTTGGGAAAGAGTTACTACATATGAAGATGGTGGTGTGTTGACTGCAACACAAGATACATCATTGCAAGATGGTT GTTTGATCTATAACGTTAAAGTTAGAGGTGTTAATTTTCCAGCTAATGGTCCAGTTATGCAAAAAGAAAACCTTTGGGTTGGGAAGC TTCTACTGAAACAATGTACCCAGCAGATGGTGGTTTGAAGGTGCTTGTGATATGGCATTGAAATGGTTGGTGGTGGTCATTTG ATCTGTAATTTGGAAACTACATACAGATCTAAGAAACCAGCTACAAATTTGAAGATGCCAGGTGTTTACAACGTTGATCATAGATT GGAAAGAATTAAGAAGCAGATGATGAAACTTACGTTGAACAACATGAAGTTGCTGTTGCAAGATACTCTACAGGTGGTGGTCTG GTGACCGTGGTAAATAA
ymScarlet	ATGGTTTCTAAAGGTGAAGCAGTTATTAAGGAATTCATGAGATTCAAGGTACACATGGAAGGTTCTATGAATGGTCACGAATTTG AAATTGAAGGTGAAGGTGAAGGTAGACCATATGAAGGTACTCAAACCTGCTAAGTTGAAGGTTACTAAAGGTGGTCCATTGCCAT TTTCTTGGGATATTTGTCTCCACAATTCATGTACGGTTCTAGAGCTTTTACAAAACATCCAGCAGATATTCCAGATTACTACAAGC AATCATTCCAGAAAGGTTTAAATGGGAAAGAGTTATGAACCTCGAAGATGGTGGTGCAGTTACTGTTACACAAGATACTTCTTT GGAAGATGGTACATTGATCTATAAGGTTAAGTTGAGAGGTACTAATTTCCACCAGATGGTCCAGTTATGCAAAAAGAAAACATG GGTTGGGAAGCTTCAACAGAAAGATTGTACCCAGAAGATGGTGTGTTTGAAGGGTGACATTAAGATGGCATTGAGATTGAAGGA TGGTGGTAGATATTTGGCTGATTTCAAGACTACATACAAGGCTAAGAAACCAGTTCAAATGCCAGGTGCTTACAACGTTGATAGA AAGTTGGATATTCTTCTCATAATGAAGATTACACAGTTGTTGAACAATATGAAAGAAGTGAAGGTAGACACAGTACAGGTGGTAT GGATGAATTATACAAATGA

Data availability

F1000Research: Dataset 1. Raw data behind [Figure 1](#), [Figure 2](#), [Figure 3](#) and [Figure 4](#), <https://doi.org/10.5256/f1000research.15829.d225385> (Albakri *et al.*, 2018)

Both p415 GPD-yFusionRed and p415 GPD-ymScarlet are available from addgene (#111916/11917). All plasmids are available upon request from the corresponding author.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Current Referee Status:  

Version 2

Referee Report 07 December 2018

<https://doi.org/10.5256/f1000research.17889.r41113>



Danny M. Hatters 

Dept of Biochem & Mol Biology, The University of Melbourne, Melbourne, VIC, 3010, Australia

I have read the revised version and I am happy with the changes the authors have made and I now recommend the work be indexed.


Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 27 November 2018

<https://doi.org/10.5256/f1000research.17889.r41114>



Fedor V. Subach 

Moscow Institute of Physics and Technology (MIPT), Moscow, Russian Federation

I have no further comments to make.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 21 August 2018

<https://doi.org/10.5256/f1000research.17277.r37082>



Fedor V. Subach 

Moscow Institute of Physics and Technology (MIPT), Moscow, Russian Federation

In this manuscript Albakri Maram et al. demonstrated superior performance of the mScarlet red fluorescent protein over the FusionRed protein in the Huntington's disease related yeast assay. First, authors showed the higher brightness of non-fused mScarlet protein vs FusionRed during constitutive expression in yeast. Albakri Maram et al. then made fusions of the mScarlet and FusionRed proteins with first exon of the Huntingtin protein (Httex1) carrying FLAG-tag and either non-pathological 25 repeats of glutamines or Huntington's disease-associated 68 repeats of glutamines. Authors expressed these fusions in yeast and found that in opposite to the FusionRed protein, mScarlet displayed right cytotoxicity and polyQ aggregation. Overall, mScarlet red fluorescent protein can be used as a right tag in polyQ yeast assay for the Huntington's disease studies. The strategy suggested in this paper may be used researchers for characterization of the properties of other newly developed fluorescent markers.

Major points:

1) Results and discussion section, page 6, right column. Analyzing protein expression level on dot blot authors revealed lower levels of yFusionRed protein as compared to other RFPs and based on this fact they suggested its increased degradation in yeast cells (actually, one cannot exclude that yFusionRed lower expression level is not related with its high degradation on RNA level not protein level). These findings suggest that lower expression level of yFusionRed protein in yeast may prevent FusionRed protein from toxicity and formation of aggregates, which could be observed at higher FusionRed protein concentrations only. So we cannot say unambiguously about the real reasons of the absence of toxicity and aggregates formation in the case of FLAG-68Q-yFusionRed fusion. Lower expression level of yFusionRed also may mask its tendency to oligomerize inherently and prevent from correct comparison in this respect with other fluorescent proteins.

Minor points:

- 1) Introduction section, page 3, left column, last sentence, please, replace misprint "...and aggregation assays to explore to effects of..." with "...and aggregation assays to explore the effects of..."
- 2) Methods section, page 3, right column, Yeast strains and culture conditions section, authors use term W303A. However according to provided reference it should be named as W303-1A. Please, correct.
- 3) Methods section, page 3, right column, DNA constructs section, authors mention that they do not use proline rich domain. Please, add explanation of the reason of this.
- 4) Methods section, page 3, right column, Dot blot section, please, replace misprint "...PSMF..." with "...PMSF..."
- 5) Methods section, page 3, right column, Dot blot section, please, replace misprint "...at room temperature were then processed..." with "...at room temperature and then processed ..."
- 6) Methods section, page 3, right column, Fluorescent microscopy section and elsewhere in the text, please, replace misprint "...Fluorescent microscopy..." with "...Fluorescence microscopy..."
- 7) Methods section, page 4, Table 1. I would recommend to change the abbreviations of the constructs in order to mention Httex1 and avoid 25Q abbreviation which can be mistakenly interpreted as a substitution of the residue in position 25 with Q, i.e. for example, replace "p415 Gal1-FLAG-25Q-ymsfGFP" with "p415 Gal1-FLAG-Httex1-(Q)25-ymsfGFP".
- 8) Methods section, page 5, Figure 2, panel A. I would recommend replace "Httex1" with "Httex1(Q)25/(Q)68".
- 9) Methods section, page 5, Figure 2, panel A and elsewhere in the text. In accordance to the 6th point I would recommend replace "25Q" and "Q68" with "(Q)25" and "(Q)68".
- 10) Methods section, page 5, Legend to the Figure 1. Please, add explanation that panel (A) represents a "Scheme of fluorescent proteins expression vectors ...". Also, please, be consistent in the same respect for the panel (C).
- 11) Methods section, page 5, legend to the Figure 2. Please, add explanation that panel (A) represents a

“Scheme of fluorescent proteins expression system ...”, panel (B) – “Image of yeast colonies ...” and panel (C) – “Dot blots of ...”.

12) Results and discussion section, page 6, left column and elsewhere in the text and figure legends, please, replace misprint “Fluorescent intensities...” with “...Fluorescence intensities...”.

13) Results and discussion section, page 6, left column, authors mention that “...yFusionRed was significantly dimmer than yemRFP”. Below they write about the strongest fluorescent signal for ymScarlet. Please, provide these comparisons in more quantitative and scientific manner, i.e. mention in how many fold yFusionRed and ymScarlet RFPs were dimmer and brighter than yemRFP, respectively.

14) Results and discussion section, page 6, left column, please, replace misprint “... in yeast can differed from ...” with “... in yeast can be different from ...”.

15) Results and discussion section, page 6, left column, please, replace misprint “... of mScarlet compared to other RFs...” with “...of mScarlet compared to other RFs ...”.

16) Results and discussion section, page 6, right column. Authors use 68 repeats of Q however in their previous paper of the year 2017 they utilized 72 Q repeats. Please, include in the text explanation why did you choose 68 not 72 repeats. It is important for the comparison of the data.

17) Results and discussion section, page 6, right column. Authors write that “...yFusionRed displayed no significant toxicity...”. The term “significant” suggests statistical analysis, could you, please, provide which statistic criterion was used in this case, e.g. a two-tailed student t-test or Mann-Whitney or someone else? Now Statistical analysis section describes Figure1D data only.

Overall, if the points raised above will be adequately addressed, indexing in F1000Research is appropriate.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 08 Nov 2018

Patrick Lajoie, University of Western Ontario

We thank the reviewer for helpful comments. We have addressed concerns in the new version of the manuscript. See below for the authors' response to specific points raised during the review process.

Major points:

1) Results and discussion section, page 6, right column. Analyzing protein expression level on dot blot authors revealed lower levels of yFusionRed protein as compared to other RFPs and based on this fact they suggested its increased degradation in yeast cells (actually, one cannot exclude that yFusionRed lower expression level is not related with its high degradation on RNA level not protein level). These findings suggest that lower expression level of yFusionRed protein in yeast may prevent FusionRed protein from toxicity and formation of aggregates, which could be observed at higher FusionRed protein concentrations only. So we cannot say unambiguously about the real reasons of the absence of toxicity and aggregates formation in the case of FLAG-68Q-yFusionRed fusion. Lower expression level of yFusionRed also may mask its tendency to oligomerize inherently and prevent from correct comparison in this respect with other fluorescent proteins.

We agree with the reviewer on this point. We have now performed additional experiments where polyQ fusions are expressed using multicopy plasmids (2 μ) where cells can express up to 50 copies of the constructs. Interestingly, under these conditions, 72Q-yFusionRed can indeed aggregate and induce toxicity, albeit to lower extent than ymsfGFP-tagged polyQ counterparts. Therefore it is reasonable to hypothesize that lower expression of yFusionRed (maybe due to increased protein turnover) is responsible for decreased toxicity. Further experiments beyond the scope of this research note will be required to explain the relative lower expression of FusionRed in yeast.

Minor points:

1) Introduction section, page 3, left column, last sentence, please, replace misprint "...and aggregation assays to explore to effects of..." with "...and aggregation assays to explore the effects of..."

We have modified accordingly.

2) Methods section, page 3, right column, Yeast strains and culture conditions section, authors use term W303A. However according to provided reference it should be named as W303-1A. Please, correct.

We have modified accordingly.

3) Methods section, page 3, right column, DNA constructs section, authors mention that they do not use proline rich domain. Please, add explanation of the reason of this.

We added the rationale for the use of the Δ proline constructs which is described in Duennwald et al., 2006 PNAS.

4) Methods section, page 3, right column, Dot blot section, please, replace misprint "...PSMF..." with "...PMSF..."

We have modified accordingly.

5) Methods section, page 3, right column, Dot blot section, please, replace misprint "...at room temperature were then processed..." with "...at room temperature and then processed ...".

We have modified accordingly.

6) Methods section, page 3, right column, Fluorescent microscopy section and elsewhere in the text, please, replace misprint "...Fluorescent microscopy..." with "...Fluorescence microscopy...".

We have modified accordingly.

7) Methods section, page 4, Table 1. I would recommend to change the abbreviations of the constructs in order to mention Httex1 and avoid 25Q abbreviation which can be mistakenly interpreted as a substitution of the residue in position 25 with Q, i.e. for example, replace "p415 Gal1-FLAG-25Q-ymsfGFP" with "p415 Gal1-FLAG-Httex1-(Q)25-ymsfGFP". 8) Methods section, page 5, Figure 2, panel A. I would recommend replace "Httex1" with "Httex1(Q)25/(Q)68". 9) Methods section, page 5, Figure 2, panel A and elsewhere in the text. In accordance to the 6th point I would recommend replace "25Q" and "Q68" with "(Q)25" and "(Q)68".

We thank the reviewer for the suggestion but the authors believe that to be consistent with the literature in the field, the current nomenclature should be used.

10) Methods section, page 5, Legend to the Figure 1. Please, add explanation that panel (A) represents a "Scheme of fluorescent proteins expression vectors ...". Also, please, be consistent in the same respect for the panel (C).

We have modified accordingly.

11) Methods section, page 5, legend to the Figure 2. Please, add explanation that panel (A) represents a "Scheme of fluorescent proteins expression system ...", panel (B) – "Image of yeast colonies ..." and panel (C) – "Dot blots of ...".

12) Results and discussion section, page 6, left column and elsewhere in the text and figure legends, please, replace misprint "Fluorescent intensities..." with "...Fluorescence intensities...".

We have modified accordingly.

13) Results and discussion section, page 6, left column, authors mention that "...yFusionRed was significantly dimmer than yemRFP". Below they write about the strongest fluorescent signal for ymScarlet. Please, provide these comparisons in more quantitative and scientific manner, i.e. mention in how many fold yFusionRed and ymScarlet RFPs were dimmer and brighter than yemRFP, respectively.

We added values in the text.

14) Results and discussion section, page 6, left column, please, replace misprint "... in yeast can differed from ..." with "... in yeast can be different from ...".

We have modified accordingly.

15) Results and discussion section, page 6, left column, please, replace misprint "... of mScarlet compared to other RFs..." with "...of mScarlet compared to other RFs ...".

We have modified accordingly.

16) Results and discussion section, page 6, right column. Authors use 68 repeats of Q however in their previous paper of the year 2017 they utilized 72 Q repeats. Please, include in the text explanation why did you choose 68 not 72 repeats. It is important for the comparison of the data.

The polyQ repeats are known to be unstable. Sequencing of the DNA revealed that our 72Q original plasmid has mutated into 68Q. This change does not impact the toxicity of the fusion. We added a note in the material and methods section.

17) Results and discussion section, page 6, right column. Authors write that "...yFusionRed displayed no significant toxicity...". The term "significant" suggests statistical analysis, could you, please, provide which statistic criterion was used in this case, e.g. a two-tailed student t-test or Mann-Whitney or someone else? Now Statistical analysis section describes Figure1D data only. ***Statistical analysis was performed on the growth curve data and described in the updated figure legends.***

Competing Interests: No competing interests were disclosed.

Referee Report 17 August 2018

<https://doi.org/10.5256/f1000research.17277.r37079>



Danny M. Hatters , **Dezerae Cox** 

Dept of Biochem & Mol Biology, The University of Melbourne, Melbourne, VIC, 3010, Australia

This study reports the impact of three different red fluorescent protein (RFP) tags on the expression and toxicity patterns of Httex1 in yeast by benchmarking them against the green fluorescent protein tagged Httex1 as the established model system in the literature. The authors reveal some differences in their effects on Httex1 toxicity and these results are likely to be of interest to those in the Httex1-polyQ field. Overall, the manuscript is well-written and figures are clearly presented. It should be noted that the phenomena reported here is a direct extension of a broader study of other fluorescent protein fusions previously reported by the same group (and has been referenced).

Major queries:

1. The authors state that the median fluorescent intensities of Fig 1 "reveals yFusionRed was significantly dimmer than yemRFP ... ymScarlet displayed the strongest fluorescent signal". As written, this implies the median fluorescence intensity provides a measure of the relative brightness of individual fluorophores. However, without quantification of the amount of protein expressed this difference in median fluorescence could simply reflect differences in total fluorophore abundance. We are not familiar with yeast transfection methods - but certainly in mammalian transfection experiments cells will invariably have a massive (many fold) variation in expression levels within populations and between different constructs including a notable population of cells that have no expression. If this is the case in yeast, then the interpretation of difference in brightness is not sufficiently supported by the data. This point needs clarification.
2. Following from the above point, it is difficult to ascertain whether the differences in expression, toxicity and aggregate extents of the various fluorescent protein tagged Htt constructs (Fig 2) arises from the same issues. To us this would be a more likely (and simpler) explanation than what the authors explain as the protein constructs having differences in inherent toxicity (molecule per molecule). Further clarification of this issue is required.
3. The level of reproducibility should be indicated by including at least 3 independent replicates for the dot blots and growth assays (in Fig 2). Also, the dot blots should be quantified with the appropriate statistical methods.

4. In Fig 1D, the authors report a significant difference in the median fluorescent intensities using a Student's t-test to compare only two of the RFPs examined. It is suggested that an ANOVA would be more appropriate because there are more than two groups of interest. Based on the data provided, our ANOVA reports a higher degree of statistical significance for the comparison currently reported (yemRFP v ymScarlet, **) and also supports a significant difference between ymFusionRed and ymScarlet (***).

Minor suggestions:

1. This sentence in the abstract has an unclear meaning as it implies ymScarlet is inherently affected by polyQ: "We found that unlike yemRFP and yFusionRed, the synthetically engineered ymScarlet displayed severe polyQ toxicity and aggregation similar to what is observed for green FP variants." Thus the sentence should be revised.
2. Additional details for the Dot blot method (secondary antibody for Pkt-1, dilutions) would enhance reproducibility
3. Include dimension for scale bar in Figure 2D

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Referee Expertise: Experts in mammalian cell biology and Httex1 mechanisms.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 08 Nov 2018

Patrick Lajoie, University of Western Ontario

We would like to thank the reviewers for their helpful comments. Below is our point-by-point response addressing the concerns raised during the review process.

Major queries:

1. The authors state that the median fluorescent intensities in Fig 1 “reveals yFusionRed was significantly dimmer than yemRFP ... ymScarlet displayed the strongest fluorescent signal”. As written, this implies the median fluorescence intensity provides a measure of the relative brightness of individual fluorophores. However, without quantification of the amount of protein expressed this difference in median fluorescence could simply reflect differences in total fluorophore abundance. We are not familiar with yeast transfection methods - but certainly in mammalian transfection experiments cells will invariably have a massive (many fold) variation in expression levels within populations and between different constructs including a notable population of cells that have no expression. If this is the case in yeast, then the interpretation of difference in brightness is not sufficiently supported by the data. This point needs clarification.

Yeast transformations are similar to E. coli transformations that the reviewers must be familiar with. All cells in the population are expressing the constructs since the cells are kept under constant selection. Since all constructs are expressed under the same promoter in a low copy centromeric vector (1-2 copies per cell), there is no reason to believe that some reason yFusionRed cells do not express fusion at the mRNA level. Quantification of the relative protein levels is problematic since anti-RFP antibodies could recognize mScarlet and yFusionred with different affinities. Addition of an additional epitope tag could affect behavior of the FPs. That said, we have used the polyQ fusions to show that yFusionRed protein levels are decreased using dot blot (see #3) and this appears to mask its ability to aggregate and consequently generate a toxic phenotype (Figure 4).

1. Following from the above point, it is difficult to ascertain whether the differences in expression, toxicity and aggregate extents of the various fluorescent protein tagged Htt constructs (Fig 2) arises from the same issues. To us this would be a more likely (and simpler) explanation than what the authors explain as the protein constructs having differences in inherent toxicity (molecule per molecule). Further clarification of this issue is required.

We agree with the reviewer on this point. We have now performed experiments where polyQ fusions are expressed using multicopy plasmids (2 μ) where cells can express up to 50 copies of the constructs. Interestingly, under these conditions, 68Q-yFusionred can indeed aggregate and induce toxicity, albeit to lower extent than ymsfGFP-tagged polyQ counterparts. Therefore it is reasonable to hypothesize that lower expression of yFusionRed (maybe due to increased protein turnover or mRNA level) is responsible for decreased toxicity. Further experiments beyond the scope of this research note will be required to explain the relative lower expression of FusionRed in yeast.

1. The level of reproducibility should be indicated by including at least 3 independent replicates for the dot blots and growth assays (in Fig 2). Also, the dot blots should be quantified with the appropriate statistical methods.

We have now added quantification of the dot blots and performed quantitative growth assays in liquid media to complement the spot assays on agar plates.

1. In Fig 1D, the authors report a significant difference in the median fluorescent intensities using a Student's t-test to compare only two of the RFPs examined. It is suggested that an ANOVA would be more appropriate because there are more than two groups of interest. Based on the data provided, our ANOVA reports a higher degree of statistical significance for the comparison currently reported (yemRFP v ymScarlet, **) and also supports a significant difference between ymFusionRed and ymScarlet (***)

We thank the reviewer and have now added appropriate statistical analysis.

Minor suggestions:

1. This sentence in the abstract has an unclear meaning as it implies ymScarlet is inherently affected by polyQ: “We found that unlike yemRFP and yFusionRed, the synthetically engineered ymScarlet displayed severe polyQ toxicity and aggregation similar to what is observed for green FP variants.” Thus the sentence should be revised.

We have modified accordingly.

1. Additional details for the Dot blot method (secondary antibody for Pkt-1, dilutions) would enhance reproducibility

We have added additional information.

1. Include dimension for scale bar in Figure 2D.

We have added the information.

Competing Interests: No competing interests were disclosed.

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