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Detecting Soluble PolyQ Oligomers and Investigating Their Impact on Living Cells Using Split-GFP

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Abstract

Aberrant expansion of the number of polyglutamine (polyQ) repeats in mutant proteins is the hallmark of various diseases. These pathologies include Huntington's disease (HD), a neurological disorder caused by expanded polyQ stretch within the huntingtin (Htt) protein. The expansions increase the propensity of the Htt protein to oligomerize. In the cytoplasm of living cells, the mutant form of Htt (mHtt) is present as soluble monomers and oligomers as well as insoluble aggregates termed inclusion bodies (IBs). Detecting and assessing the relative toxicity of these various forms of mHtt has proven difficult. To enable direct visualization of mHtt soluble oligomers in living cells, we established a split superfolder green fluorescent protein (sfGFP) complementation assay. In this assay, exon 1 variants of Htt (Htt^{ex1}) containing non-pathological or HD-associated polyQ lengths were fused to two different nonfluorescent fragments of sfGFP. If the Htt proteins oligomerize and the sfGFP fragments come into close proximity, they can associate and complement each other to form a complete and fluorescent sfGFP reporter. Importantly, the irreversible nature of the split-sfGFP complementation allowed us to trap otherwise transient interactions and artificially increase mHtt oligomerization. When coupled with a fluorescent apoptosis reporter, this assay can correlate soluble mHtt oligomer levels and cell death leading to a better characterization of the toxic potential of various forms of mHtt in living cells.

Keywords

Split-GFP; Huntingtin exon 1; Oligomers; Cell death

1 Introduction

Huntington's disease occurs due to expansion of CAG repeats in the huntingtin (Htt) gene, which increases the number of polyglutamine (polyQ) repeats and tendency of mutant Htt (mHtt) protein to aggregate in cells. Asymptomatic individuals usually present fewer than 36 CAG repeats [1]. The age of onset of the pathology inversely correlates with the number of repeats [2]. mHtt aggregation leads to the formation of insoluble inclusion bodies (IBs). The role and consequences of IBs remain controversial. While IBs have been proposed to be toxic to neurons [3–5], other studies report evidence for IBs as a cellular coping mechanism to manage the accumulation of mutant proteins by reducing levels of soluble and potentially toxic monomeric and oligomeric species [6, 7]. Full-length Htt is a large protein (3,144

amino acids) associated with low rates of aggregation in vitro. Indeed, transgenic mice expressing the full-length version of Htt show little impact on the animal longevity with a weak HD phenotype [8]. In contrast, introduction of exon 1 (the first 67 amino acids plus internal variable polyglutamine stretch) of the HD gene in transgenic mice is sufficient to cause a progressive HD phenotype in as little as 3 weeks [9]. In the absence of early biomarkers for HD, the exon 1 model remains a useful alternative to study Htt oligomerization in cell culture.

To determine which form(s) of mHtt is responsible for neuronal cell death in HD, different approaches have been used to correlate formation of soluble oligomers with cell death. In this chapter, we describe a protocol to visualize mHtt oligomers in living cells using split-GFP [10]. In this assay, a fluorescent protein, i.e., superfolder GFP (sfGFP) [11], is split into two nonfluorescent fragments and independently fused to Htt^{ex1} containing either a non-pathological or an HD-associated length of polyQ. When the Htt proteins oligomerize and if the sfGFP fragments come into close enough proximity, molecular complementation between the two nonfluorescent fragments results in the formation of fluorescent reporter [12, 13]. The irreversible nature of this association allows the trapping of transient protein–protein interactions [14]. The trapping leads to an artificial increase in mHtt^{ex1} soluble oligomers [13]. When used in conjunction with cell death reporters, split-sfGFP (or other fluorescent variants) reporters are powerful tools for studying the impact of polyQ oligomers on cell survival.

2 Materials

2.1 Generation of Htt^{ex1} Split-sfGFP Constructs

1. cDNA encoding Htt^{ex1} sequences containing various numbers of polyQ repeats.
2. Vector encoding monomeric superfolder GFP.
3. PCR thermocycler.
4. Reagents for PCR reaction: dNTP mix, Taq polymerase, primers.
5. Restriction enzymes and appropriate buffers.
6. Agarose gel apparatus.
7. Gel and PCR purification kits.
8. T4 ligase and buffer.
9. Competent DH5α bacteria for transformation.
10. DNA purification kit such as a mini prep kit.

2.2 Expression of Htt^{ex1} Split-sfGFP Constructs in Mammalian Cells

1. Laminar flow tissue culture hood and basic tissue culture material.
2. Multi-well Labtek chambers (Thermo Fisher, Pittsburgh, PA).
3. Phenol red-free complete Roswell Park Memorial Institute medium (RPMI) (containing L-glutamine, penicillin/streptomycin, and 10 % fetal bovine serum).

4. Transfection reagent such as Lipofectamine (Life Technologies, Carlsbad, CA).
5. Htt^{ex1} split-sfGFP plasmids and ER-DEVD-tdTomato ODC plasmid.
6. Fluorescence microscope.

2.3 Labeling of Htt^{ex1} Oligomers by Immunofluorescence

1. Formaldehyde solution (37 %), less than 6 months old since opening.
2. 10 % (v/v) fetal bovine serum (FBS) in phosphate buffered saline (PBS).
3. Anti-GFP (raised against the N-terminal part of GFP) and anti-myc primary antibodies (from different hosts, i.e., mouse and rabbit).
4. Fluorescently conjugated secondary antibodies (such as Alexa fluorophore-conjugated antibodies from Life Technologies, Carlsbad, CA).
5. 0.1 % (w/v) Triton X-100 in PBS.

2.4 Quantitation of Htt^{ex1} Split-sfGFP Oligomers in Living or Fixed Cells

1. Image processing software, such as the freely available ImageJ (<http://rsbweb.nih.gov/ij/>).

3 Methods

3.1 Generation of Htt^{ex1} Plasmids

1. The first step is to design primers to amplify the Htt^{ex1} fragments. In our protocol, we cloned the Htt^{ex1} sequences (Fig. 1a) containing 23, 73, and 145 polyQ (Coriell Institute, Camden, NJ) to the BglII/AgeI sites of pEGFP-N1 vector (Clontech, Mountain View, CA) using primers described in Fig. 1b. Note that we generated the Htt^{ex1} fragments with and without a myc epitope tag (EQKLISEEDL) for future differential recognition by immunofluorescence.
2. Using these primers, prepare the following PCR reaction mix:
 - 5 µl 10× PCR buffer
 - 41 µl water
 - 1 µl Primer mix (dilute at 1 µl 100 µM Forward primer + 1 µl 100 µM Reverse primer + 3 µl dH₂O)
 - 1 µl dNTPs
 - 1 µl template (10 ng/µl stock)
 - 1 µl Pfu polymerase (2.5 U/µl)
 - 50 µl
3. Amplify using standard PCR protocol.
4. Run the resulting PCR product on a 1 % agarose gel.

5. Purify the PCR product using a commercial gel purification kit (such as Qiagen, Valencia, CA).
6. Digest the PCR fragment and the pEGFP-N1 vector using BglII/AgeI enzymes according to manufacturer's instructions.
7. Ligate the digested PCR fragment into the digested vector according to the manufacturer's instructions.
8. Transform into DH5 α competent bacteria according to manufacturer's instructions and plate into agar plate containing kanamycin (if using a kanamycin resistance vector).
9. Pick a colony and inoculate into 5 ml of LB broth and grow overnight.
10. Purify the plasmid using commercial kit.
11. Verify successful cloning by diagnostic digestion and sequencing.

3.2 Integrate the sfGFP and Split-sfGFP Fragments into the Htt^{ex1} Plasmids

We used the AgeI/NotI sites of the pEGFP-N1 vector.

1. The first step is to design primers to amplify the different sfGFP fragments. The split-sfGFP first fragment contains the first 157 amino acid of sfGFP (s157) while the second one contains the remaining sequence of the protein (s238) (Fig. 1c). Primers to generate the full-length sfGFP protein should also be designed (Fig. 1).
2. Repeat **steps 2–11** of the previous Subheading 3.1 using appropriate buffers and restriction enzymes.

3.3 Quantitative Imaging of Htt^{ex1} Split-sfGFP Plasmids Oligomerization by Immunofluorescence

First, the ability of both Htt^{ex1} plasmid constructions to complement and produce a fluorescent GFP signal must be verified.

1. Plate Neuro2a cells (N2a) (*see* Note 1) into 8-well Labtek imaging chambers.
2. Transfect cells with Htt^{ex1} split-sfGFP plasmids using Lipofectamine 2000 (or your favorite transfection reagent or method) according to manufacturer's instructions. Cells should be transfected with Htt^{ex1} s157 and s238 plasmids separately (a negative control) or in combination. A second set of negative controls is to transfect s157 and s238 sfGFP plasmids that do not have Htt insertions, together or separately or the complementary nonfused version along with the Htt^{ex1} fusion construct. Yet another useful control is to test interactions with another irrelevant cytoplasmic protein (such as Nalp1b) fused to one of the split-sfGFP fragments. Together these controls will establish the specificity of the polyQ-split-GFP interaction. Finally, it is also important to test whether the position of the split-GFP fragment influences the complementation. This can be determined by generating a

¹N2a cells can be routinely differentiated into neuron-like cells by incubating the cells with 5 μ M dbcAMP (N69, 29-O-dibutyrylaenosine-39:59-cyclic monophosphate sodium salt) (Sigma-Aldrich, St. Louis, MO) for 2 days.

plasmid with an Htt^{ex1} protein with a split-sfGFP fragment fused to its NH₂-terminal end.

3. Grow cells for 48 h in complete RPMI media.
4. Fix cells in freshly diluted 3.7 % formaldehyde in PBS containing for 15 min at room temperature.
5. Permeabilize cells using 0.1 % (w/v) Triton X-100 in PBS for 15 min at room temperature.
6. Prevent nonspecific antibody binding by blocking epitopes in cells with 10 % (v/v) FBS in PBS for 1 h at room temperature.
7. Incubate cells with 200 µl of 10 % (v/v) FBS in PBS containing anti-GFP and anti-myc antibodies for 1 h.
8. Wash 3× 10 min with 400 µl of PBS 10 % (v/v) FBS in FBS.
9. Incubate cells with 200 µl of 10 % (v/v) FBS in PBS containing appropriate fluorescently labeled secondary antibodies for 1 h.
10. Wash 3× 10 min with PBS.
11. Observe cells under the fluorescent microscope. No fluorescent signal should be detected when split-GFP fragments are expressed separately. For each fluorescence channel (anti-GFP, anti-myc, and sfGFP fluorescence), set image acquisition settings so the brightest conditions are not saturated (in our case the Q73 s157/Q73 s238) and use the same settings for all different conditions (*see* Note 2).
12. Quantitate the fluorescence signal of each channel in ImageJ. This can be done by manually tracing the individual cells as close to the edge as possible for maximum signal and then measuring the average pixel fluorescence intensity for each cell.
13. Plot the average fluorescent intensities for the different conditions (Fig. 2).

3.4 Quantitative Imaging of Htt^{ex1} Split-sfGFP Plasmids Oligomerization and IBs Formation in Living Cells and Impact on Cell Death

1. Plate Neuro2a cells (N2a) (or any other adherent cell type of interest) into 8-well Labtek imaging chambers.
2. Transfect cells with Htt^{ex1} split-sfGFP or intact sfGFP plasmids using Lipofectamine 2000 according to manufacturer's instructions (or favorite transfection method). During the transfection step, a live cell apoptosis reporter such as the ER-DEVD-tdTomato ODC [13, 15] can be incorporated (Fig. 3) (*see* Note 3).

²When wanting to directly compare fluorescent intensities between two different condition, it is critical that the two images were acquired with the same acquisition settings. To avoid pixel saturation, we routinely acquired the brightest image first and keep the same settings to acquire the other conditions. For the polyQ constructs, because of the very intense signal originating from the IBs, it may be useful to acquire two different images (with high and low exposures) to visualize and measure a nonsaturating cytoplasmic GFP signal.

³The reporter localizes to the endoplasmic reticulum (ER). Upon induction of apoptosis with staurosporine, activated caspases cleave the DEVD peptide in the reporter, releasing it from the ER membrane and allowing its translocation into the nucleus.

3. Incubate cells in complete RPMI media.
4. At various time intervals, observe cells under the fluorescent microscope. For the GFP fluorescence channel, set image acquisition settings so the brightest conditions are not saturated and use the same settings for all different conditions (*see* Note 2). At the same time, record the percentage of cells expressing IBs.
5. Plot the percentage cells expressing IBs for both full sfGFP and split-sfGFP Htt^{ex1} constructs over time (Fig. 4).
6. Quantitate the fluorescent signal of each channel in ImageJ. This can be done by manually tracing the individual cells, as close to the cell edge as possible, and measuring the mean fluorescence intensity (pixel intensity) for each cell.
7. Quantitate DEVDase activity in individual cells by dividing the mean nuclear fluorescence intensity of the ER-DEVD-tdTomato ODC by the ER mean fluorescence intensity.
8. Plot the average fluorescence intensities for the different conditions as a function of DEVDase activity (Fig. 5).

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a

Htt^{ex1} Q23 sequence:

[illegible]

MATLEKLMKAFESLKSFQQQQQQQQQQQQQQQQQQQQQQPPPPPPPPPPQLPQPPPPQA
QPLLQPQPPPPPPPPPPGPAVAEEPLHRP

b

Primer used:

Htt^{ex1} fragments can be amplified by PCR using the following primers:

forward primer GATCAGATCTGCCACCATGGCGACCCTGGAAAAG

reverse primer GATCACCGGTCCTGGTCGGTGCAGCGG

and subcloned into the BglII and AgeI site of monomeric msfGFP-N1 vector.

Split-sfGFP constructs can be generated by PCR using msfGFP-N1 as a template with the following primers:

s157

forward primer 5' GCAAATGGGCGGTAGGCG 3'

reverse primer 5' GATCGCGGCCGCTTACTGCTTGTCGGCCATG 3'

and subcloned into Age1 and Not1 of SFpEGFP-N1.

S238

forward primer 5' GATCACCGGTCCGGGAGCAAGAACGGCATCAAG 3'

reverse primer 5' GGTTCAGGGGGAGGTGT 3'

and subcloned into Age1 and Not1 of msfGFP-N1

C

mSFGFP (s157 in bold, s238 underlined)

MYSKGEELFTGVVPLVELDGDVNGHKFSVRGEGEDATNGKLTCLKICTTGKLPVPWPT
 LVTTLLYGVQCFSRYPDHMKRHDFFKSAMPEGYQERTISFKDDGTYKTRAEVKFEGDTL
 VNRIELKIGDFKEDGNILGHKLEYSFNSHNVYITADKQKNGIKANFKIRHNVEDGSLVA
 DHYQONTPIDGEPVLLPDNHYLSTOVSLSKDPNKRDKMVLLEKRVTAAGITHMGDELTY*

ATGGTGAGCAAGGGCGAGGAGCTGTTCAACGGGGTGGTGCCATCCTGGTCGAGCTGGAC
GGCGACGTAAACGGCCACAAGTTTACGCGTGC GCGGCGAGGGCGAGGGCGATGCCACCAAC

GGCAAGCTGACCCTGAAGTTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCACC
 CTCGTGACCACCTCTGACCTACGGCGTGACGTGCTTCAGCGCGTACCCCGACCATATGAAG
 CGCCACGACTTCTTCAAGTCCGCGTACGCCGTAAGGCTACGCTCAGGAGCGCACCATCATG
 TCAAGGACGACGCGCACTCAAGACC CGCGCAGGTGAAGTTTCAGGGCGACACCTCG
 GTGAACCGCATCGAGCTGAAGGCATCGACTTCAAGGAGGACGGCAACATCTGGGGCAC
 AAGCTGGAGTACAACCTTCAACAGCCACACGCTTATATCACCGCGCAGCAAGAGAAAC
 GGCATCAAGGCGAACTTCAAGATTCGCGCAACACTGGAGGACGCGCAGCTGCAGCTGCC
 GACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCAC
 TACCTGAGCAGCCAGTTCGCTGCTGAGGAAGACCCCAACGAGAGCGGATACATGGTCT
 CTGCTGGAACGCGTGACCGCCGCCGGGATCACTACGGCATGACGAGCTGTACAAG

Fig. 1.

(a) Htt^{ex1} Q23 nucleotide and amino acid sequences. **(b)** Primers used to generate Htt^{ex1}-sfGFP and split-sfGFP plasmids. **(c)** Monomeric sfGFP nucleotide and amino acid sequences. s157 is highlighted in *bold* and s238 is *underlined*

Fig. 2.

Visualization of Htt^{ex1}-sfGFP oligomers using split-GFP. **(a)** Illustration of the fusion of wt and mHtt^{ex1} to either 157-GFP or 238-GFP. **(b)** Cells transfected with Q23 s157 or Q23 s238 separately, stained with anti-GFP or anti-myc, and imaged for GFP fluorescence intensity (sfGFP int.). **(c)** Representative images of N2a cells transiently transfected with Htt^{ex1}Q23 157-sfGFP+Htt^{ex1}Q23 238-sfGFP, Htt^{ex1}Q73 157-sfGFP+mHtt^{ex1}Q73 238-sfGFP, mHtt^{ex1}Q145 157-sfGFP+mHtt^{ex1}Q145 238-sfGFP, Nalp1b-157+Htt^{ex1}Q23 238-GFP, or mHtt^{ex1}Q73 157-sfGFP+238-sfGFP mHtt^{ex1}Q73. **(d)** Mean fluorescence intensities in cells without IBs are plotted and compared relative to Q23 means, which were set as 100 % (Reproduced from [13] with the permission of the Public Library of Science)

Fig. 3.

Validation of the ER-DEVD-tdTomato reporter functionality. N2a cells were transfected with ER-DEVD-tdTomato for 16 h and then treated with or without 5 μ M staurosporine for 3 h. The fluorescent intensity ratio of the nucleus over the ER calculated is presented in the plot. $** < 0.0001$ compared to untreated cells. Bar = 20 μ m (Reproduced from [13] with the permission of the Public Library of Science)

Fig. 4. Quantitation of percentage of cells containing IBs for indicated times post-transfection with Htt^{ex1} Q23, 73, or 145 fused to sfGFP or split-sfGFP constructs. $n > 215$ cells. $*p < 0.05$, $**p < 0.005$ compared to same length of polyQ fused to sfGFP (Reproduced from [13] with the permission of the Public Library of Science)

Fig. 5.

Levels of soluble cytoplasmic mHtt^{ex1} are a negative predictor of cell death. N2a cells were cotransfected with Q23, 73, or 145 Htt^{ex1} fused to sfGFP or split-sfGFP and ER-DEVD tdTomato for 48 h. GFP or split-sfGFP intensities and the nuclear/ER ratio of the apoptosis reporter were quantified for individual cells not presenting IBs. In each plot, the cell with the brightest GFP intensity was defined as having a mean intensity of 100 arbitrary units and other cell intensities were converted to this scale. Thus intensities in one plot are not directly comparable to intensities in another plot. Each square represents a single cell. Increasing mHtt^{ex1}, but not wt Htt^{ex1}, levels above some threshold were associated with increased cell death. The existence of apparent thresholds correlating with activation of the caspase activity suggests mHtt^{ex1} toxicity is sharply concentration dependent and may depend on titration of one or more key cellular factors (Reproduced from [13] with the permission of the Public Library of Science)