Video Article

Assay Development for High Content Quantification of Sod1 Mutant Protein Aggregate Formation in Living Cells

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that can be caused by inherited mutations in the gene encoding copper-zinc superoxide dismutase 1 (SOD1). The structural instability of SOD1 and the detection of SOD1-positive inclusions in familial-ALS patients supports a potential causal role for misfolded and/or aggregated SOD1 in ALS pathology. In this study, we describe the development of a cell-based assay designed to quantify the dynamics of SOD1 aggregation in living cells by high content screening approaches. Using lentiviral vectors, we generated stable cell lines expressing wild-type and mutant A4V SOD1 tagged with yellow fluorescent protein and found that both proteins were expressed in the cytosol without any sign of aggregation. Interestingly, only SOD1 A4V stably expressed in HEK-293, but not in U2OS or SH-SY5Y cell lines, formed aggregates upon proteasome inhibitor treatment. We show that it is possible to quantify aggregation based on dose-response analysis of various proteasome inhibitors, and to track aggregate-formation kinetics by time-lapse microscopy. Our approach introduces the possibility of quantifying the effect of ALS mutations on the role of SOD1 in aggregate formation as well as screening for small molecules that prevent SOD1 A4V aggregation.

Video Link

The video component of this article can be found at https://www.jove.com/video/56425/

Introduction

Protein aggregation is a biological process by which misfolded proteins group up and may act as causative agents in neurodegenerative diseases (amyloidosis). Characterizing protein aggregation is essential in understanding the role of aggregates in cellular dysfunction as well as in facilitating the discovery of new factors that influence the onset of the pathology. The visualization of fluorescence-tagged proteins in living cells is a powerful method which may aid in the development of assays applicable to high content screening (HCS)[1,2,3,4].

Amyotrophic lateral sclerosis (ALS) is regarded as a proteopathic disease caused by the presence of misfolded proteins with the propensity to aggregate and accumulate in motor neurons in both familial ALS (fALS) and sporadic ALS (sALS)[5,6]. A subset of ~20% of fALS cases are associated with dominant mutations in the gene encoding the cytosolic antioxidant enzyme copper-zinc superoxide dismutase type 1 (SOD1)[7,8]. Several potential causes for this genetically derived dysfunction have been proposed, including changes in the structure and function of SOD1 variants, such as aberrant stability, increased unfolding rate, and propensity to aggregate[9,10]. Notably, the only verified and potentially toxic property shared by both ALS-linked SOD1 variants and wild-type (WT) SOD1 is an increased propensity to form compartmentalized protein aggregates or proteinaceous inclusions[11,12]. Misfolded mutant SOD1, is persistently polyubiquitinated and degraded by the ubiquitin-proteasome system. As a result, a low level of inhibition of proteasome activity leads to the accumulation of mutant SOD1 aggregates[13,14], which form amorphous structures composed of soluble components that can exchange with soluble mutant SOD1 in the cytosol[15]. In particular, SOD1 mutant A4V (alanine at codon 4 changed to valine) is the most common ALS-causing mutation, and leads to rapid neurodegeneration with an average survival time of less than 2 years after disease onset[16]. Biochemically, SOD1 A4V has an increased tendency to monomerize, aggregate, and form amyloid pores; its pore-like aggregates are similar to amyloid pores of other disease-linked mutant forms, such as α-synuclein and β-amyloid protein[17]. To study the dynamics of SOD1-aggregate accumulation, methods for monitoring soluble and insoluble SOD1 aggregate forms remain to be developed.

We have previously shown, using live-cell imaging and HEK-293 cells transiently transfected with fluorescent protein-tagged SOD1, that ALS-associated mutations impair SOD1 dimerization and aggregation[11]. Although transient expression systems can provide useful information about the biological outcome of short-term gene overexpression, methods providing stable integration of desired genes may be preferred for assay development. As such, lentiviral vectors offer the ability to confer long-term and regulated gene expression on mammalian cells [18]. In this study, we focused on the generation of stable cell lines transduced with recombinant lentivirus bearing WT and mutant SOD1 tagged with yellow

fluorescent protein (YFP). Using live-cell imaging microscopy and automated quantification of SOD1 aggregation, we triggered and quantified SOD1 aggregation events upon inhibition of the proteasome.

Protocol

1. Lentivirus production

NOTE: The production and manipulation of lentiviral vectors was carried out according to the National Institutes of Health (NIH) guidelines for research involving recombinant DNA. The plasmid encoding the wild-type and A4V mutant SOD1 tagged with enhanced YFP (SOD1WT-YFP and SOD1A4V-YFP) are described in Kim *et al.*[11] Both gene fusion products were amplified using the PCR primer pair 5′-ATCGTCTAGACACCATGGCGACGAAGGTCGTGTGC-3′ and 5′-TAGCGG CCGCTACTTGTACAGCTCGTCCATGCC-3′and inserted into the pTRIP-delta U3 CMV plasmid[19] using XhoI and BsrGI restriction sites. In prior experiments, the HEK-293T cells were split at a ratio of 1:4 to 1:6 every two days. Avoidance of more than 20 passages and maintenance of cells at less 60% confluency helps to ensure good transfection efficiencies.

1. On Day 1, split HEK-293T cells at 30 - 40% confluence in a 10-cm diameter tissue culture plate (3 x 10^6 cells/dish) in 10 mL of cell culture medium (high-glucose DMEM with 10% FBS and 4 mM L-glutamine) for virus production.
2. Keep the 10-cm diameter tissue culture plate in a CO_2 incubator (37 °C, 5% CO_2) overnight.
3. On Day 2, prepare a DNA transfection solution containing 10 µg of lentiviral vectors (pTRIP-delta U3 CMV - SOD1WT-YFP or -SOD1A4V-YFP) together with the lentiviral packaging plasmids (5 µg of pVSVg; 10 µg of pCMV-dR8.71) (**Figure 2A**)[20]. Add 125 µL of 1 M $CaCl_2$ and adjust the volume to 500 µL using distilled water. Gently add 500 µL of 0.05 M HEPES into the mixture and incubate for 10 min at room temperature.
4. Replace HEK-293T cells medium with 10 mL pre-warmed fresh culture medium antibiotic-free mixed with 1 mL DNA transfection solution and incubate overnight at 37 °C, 5% CO_2.
5. On Day 3, replace the cell supernatant with fresh culture medium.
6. On Day 4, harvest the supernatant in a 15 mL tube and centrifuge for 5 min at 500 x g to remove the dead cells and debris. Further purify the supernatant by passing it through a 0.45 µm filter using a large 60 mL syringe. Immediately dispense into single-use aliquots (300 µL), and store at -80 °C. To maintain maximum product activity, avoid a freeze-thaw cycle.

2. Lentiviral transduction

1. Split the cell line to be infected at 60% confluence (HEK-293 cells and SH-SY5Y; 5 x 10^5 cells per well and U2OS; 1 x 10^5 cells per well) in a 6-well tissue culture plate in 2 mL of DMEM media supplemented with 10% FBS.
2. Keep the 6-well tissue culture plate in a 37 °C incubator at 5% CO_2 overnight.
3. Remove the frozen lentivirus from the -80 °C freezer and thaw an aliquot on ice before each use; do not refreeze.
4. While the virus is thawing, warm the cell culture medium containing the serum compatible with the cell line of interest. Once the virus is fully thawed, prepare a range of dilutions (1:3, 1:10, 1:30, and 1:100) in DMEM in a fresh 1.5 mL microfuge tube.
5. Bring up the volume in the tubes to 1 mL with reduced serum media.
6. Add 2 µL of Polybrene (at a stock of 4 µg/µL) to 1 mL of virus/media. Mix well by pipetting and add 1 mL of mixture to the cells. Incubate the cells with the virus for 24 h.
7. Remove the virus media and replace with normal DMEM media supplemented with 10% FBS. Maintain the cells at 37 °C, 5% CO_2.
8. Monitor the growth of the cells and change the culture media every two days. At confluence, expand the 6-well dish into a 10-cm diameter tissue culture plate.
9. Once the cells have been sufficiently expanded, seed 1.5 x 10^4 cells per well in 50 µL of DMEM media on 384-well assay plates to check the expression level of the YFP tagged protein by microscopy. If the YFP tagged protein cell expression shows a signal-to-noise ratio ≥3, prepare cell stock for the corresponding cell line.

3. Time dependent effect of proteasome inhibitor on protein aggregation

NOTE: Perform image acquisition using an automated microscope (see **Materials**).

1. Dispense 0.25 µL of DMSO or proteasome inhibitor dissolved in 100% DMSO (ALLN, 2 mM) on 384-well flat bottom plates.
2. Manually seed 1.5 x 10^4 cells per well in 50 µL of DMEM media on the same assay plate. The proteasome inhibitor (ALLN) final concentration should be 10 µM.
3. Set up the microscope system environmental control unit to 37 °C and 5% CO_2. A screenshot of the experimental setup is shown in **Figure 3**.
4. Operate the microscope in wide field fluorescence mode, using the software with the following settings: LWD 20X objective, non-confocal mode, 4 fields/well, with a capture interval of an hour. At the end of each run, captured images are automatically uploaded to the server.

4. Time lapse imaging for YFP expression in living cells, and image analysis (single channel)

NOTE: The following steps describe application of the software (*e.g.*, Columbus).

1. Select the appropriate algorithm to segment the primary objects (cytosol and aggregates). If required, adjust background threshold and contrast parameters (**Figure 4**).

2. Count cells using 'find cells' with an individual threshold of 0.1 for the YFP intensity channel. Determine aggregates using a 'find spots' algorithm with a relative YFP spot intensity >0.2 and a splitting coefficient of 1.0.

5. Dose response effect of proteasome inhibitors on protein aggregation on living cells stained for their nuclei (two channels)

NOTE: Perform image acquisition using an automated microscope. Determine the concentration range of proteasome inhibitors (ALLN, Epoxomicin, and MG132) based on the expected IC_{50} value to ensure an optimal curve fit.

1. Prepare serial dilutions of compounds on a 384-well storage polypropylene plate by the standard 1:3 dilution series. Make sure to mix the compound dilutions well to ensure that the compound concentrations are accurate.
2. Dispense 0.25 µL of proteasome inhibitors on empty flat-bottom black 384-well assay plates. We use a liquid handler equipped with a 384-capillary head capable of transferring volumes.
3. Seed 1.5 x 10^4 cells per well in 50 µL of DMEM Media on assay plates. Incubate the assay plates at 37 °C and 5% CO_2 for 24 h.
4. Add 10 µL of DMEM Media (pre-warmed at 37 °C) with staining solution (Hoechst 33342 at a stock of 10 mg/mL) and incubate at room temperature for 10 min.
5. Launch the microscope operating software. A screenshot of the experimental setup is shown in **Figure 6**. Select the "Configuration" tab and select 20X objective and the correct plate type. Ensure "collar" is set to the correct value on the objective allowing proper focus with different plate types.
6. Select the "Microscope" tab. Define exposure 1 as YFP (488 laser) and exposure 2 as Hoechst (405 laser). Activate the filter on both exposures and assign exposure 1 to camera 1 and exposure 2 to camera 2. Set exposure times to ~800 ms for exposure 1 and ~40 ms for exposure 2.
7. Select exposure 1. Set focus height to 0 µm. Select "Focus". Once focused, expose camera 1. Adjust the focus height to optimize the exposure plane and click on "Take height". Change the exposure times and laser power to give a maximum pixel intensity of ~3,000. Save exposure parameters. Repeat for exposure 2.
8. Select "Experiment Definition" tab. Create a layout and sublayout. Drag and drop the relevant layout, exposure, reference image, skewcrop file, and sublayout. Save the experiment.
9. Select "Automatic Experiment" tab and acquire images. At the end of each run, captured images are automatically uploaded to the server.

6. Image analysis of two channel images

1. Select the software algorithm to segment the primary objects (nuclei, cytosol, and aggregates) (**Figure 7**).
2. Select the method that segments nuclei accurately by visual inspection of the segmented objects. Hoechst-stained objects in channel 1 (Ch1) will be used to determine the number of cells. The YFP staining from the mutant SOD1-A4V in channel 2 (Ch2) will be used to determine the amount of aggregates.
3. Count cells using the 'find nuclei' algorithm as Hoechst-staining regions >20 μm^2, with a split factor of 7.0, an individual threshold of 0.40, and a contrast >0.10.
4. Create the data table and determine EC_{50} for each of the compounds using the 'Non-linear regression' equation in the graphing software.

Representative Results

Generating stable cell line using lentivirus: The overall strategy for monitoring SOD1 protein aggregates is illustrated in **Figure 1**. In a first step, we generated a lentiviral expression vector for SOD1 stable gene delivery into cell lines (Step 1). Two lentiviral vectors encoding YFP-tagged SOD1 WT and SOD1 A4V (SOD1WT-YFP and SOD1A4V-YFP, respectively) with packing and envelope plasmids were prepared (**Figure 2A**). Upon lentiviral transduction (Step 2) the cell lines tested (HEK-293, U2OS, and SH-SY5Y) remained healthy, showed high expression levels (**Figure 2B**), and long-term YFP labeling irrespective of the SOD1 form. Interestingly, neither WT nor mutant SOD1 expressed in the three cell lines which showed visible aggregation, in contrast to the transient expression cellular model previously tested[11].

Validating stable cell lines for SOD1 aggregation and studying its dynamic: To trigger SOD1 aggregate formation upon cellular accumulation of misfolded SOD1 mutant, we used proteasome inhibitor ALLN (also called calpain I and II inhibitor; Ki = 190 and 220 nM respectively), previously validated using a transient SOD1 expression cellular model[11]. SOD1 aggregation was examined with HEK-293, U2OS, and SH-SY5Y cell lines transduced with SOD1WT-YFP and SOD1A4V-YFP. Treatment with proteasome inhibitor ALLN (10 µM) for 24 hours strongly promoted the accumulation of SOD1A4V-YFP aggregates in HEK-293 cells (**Figure 2C**). However, no aggregation was found in SOD1WT-YFP transduced cell lines and very low levels of aggregation was found in U2OS and SH-SY5Y cell lines SOD1A4V-YFP transduced SOD1WT-YFP and SOD1A4V-YFP (**Figure 2C**). Based on these observations, we used time-lapse microscopy to investigate the dynamic of SOD1A4V-YFP aggregation formation induced by proteasome inhibition (Step 3, **Figure 3**) and to quantify SOD1A4V-YFP aggregation using a single YFP fluorescence channel to detect the expressing cells and aggregates in living cells (Step 4, **Figure 4**). Cells were seeded in the presence and absence of ALLN, and monitored for a period of 50 hours (**Figure 5A**). Under our experimental conditions, we found the aggregate formation induced by ALLN reached a plateau at 24 hours and remained stable over the next 12 hours. We show that cell density significantly decreased after 28 hours as a result of the cytotoxic effect of ALLN, while cell number increased in the DMSO-treated negative experiment.

Characterizing small molecule EC_{50} for aggregation formation using SOD1 cell model: Cells expressing SOD1A4V-YFP were treated in a dose-dependent manner with small molecule proteasome inhibitors. We used a nuclear marker to label the SOD1A4V-YFP cell line, which is advantageous for aggregate detection within the cytosol compartment. Indeed, since each cell contains a nucleus, by segmenting the nuclei and using them as seeds in watershed segmentation of the cells, cytoplasm segmentation is simplified[21]. We used Hoechst staining, which is known to have no toxicity when used for a short term period and at low concentrations[22], conditions which were not used in the previous time lapse imaging experiment. After 24 hours of cell culturing in the presence of proteasome inhibitors, SOD1A4V-YFP cells were stained with Hoechst solution (10 µg/mL) for 10 min. Next we acquired fluorescence images for Hoechst and YFP using a 20X 0.4 NA objective (Step 5, **Figure 6**). Using an image analysis algorithm which takes into account the cell nuclei and SOD1A4V-YFP distribution, cells were analyzed using the image analysis software (Step 6, **Figure 7**). Hoechst-stained objects exhibiting the appropriate fluorescent intensity above background and the morphological size characteristics of a nuclei (width, length, and area) were identified and classified by image segmentation. The nuclear mask derived from Hoechst staining was used to track the proximal SOD1A4V-YFP spot distribution within the cell cytosolic area and to determine the presence or absence of aggregates. Based on nuclei and spots detection, a ratio of aggregates detected versus cells was calculated. To determine the sensitivity of our assay, we next quantified aggregation of SOD1A4V-YFP HEK-293 cells treated with three different proteasome inhibitors (ALLN, MG132, and epoxomicin) in a dose concentration manner which enabled fitting the quantified data and we calculated EC_{50} values of 6.53 ± 1.17, 0.17 ± 0.06, and 0.03 ± 0.03 µM for ALLN, MG132 and epoxomicin, respectively (**Figure 8B**). The relative toxicity for all three proteasome inhibitors was quantified by measuring the number of adherent cells remaining in the well labeled with Hoechst dye. We found that the total cell number tended to decrease in response to compound effects, as evidenced by similar EC_{50} values of 6.58 ± 1.06, 0.19 ± 0.03, and 0.05 ± 0.01 µM for ALLN, MG132 and epoxomicin, respectively.

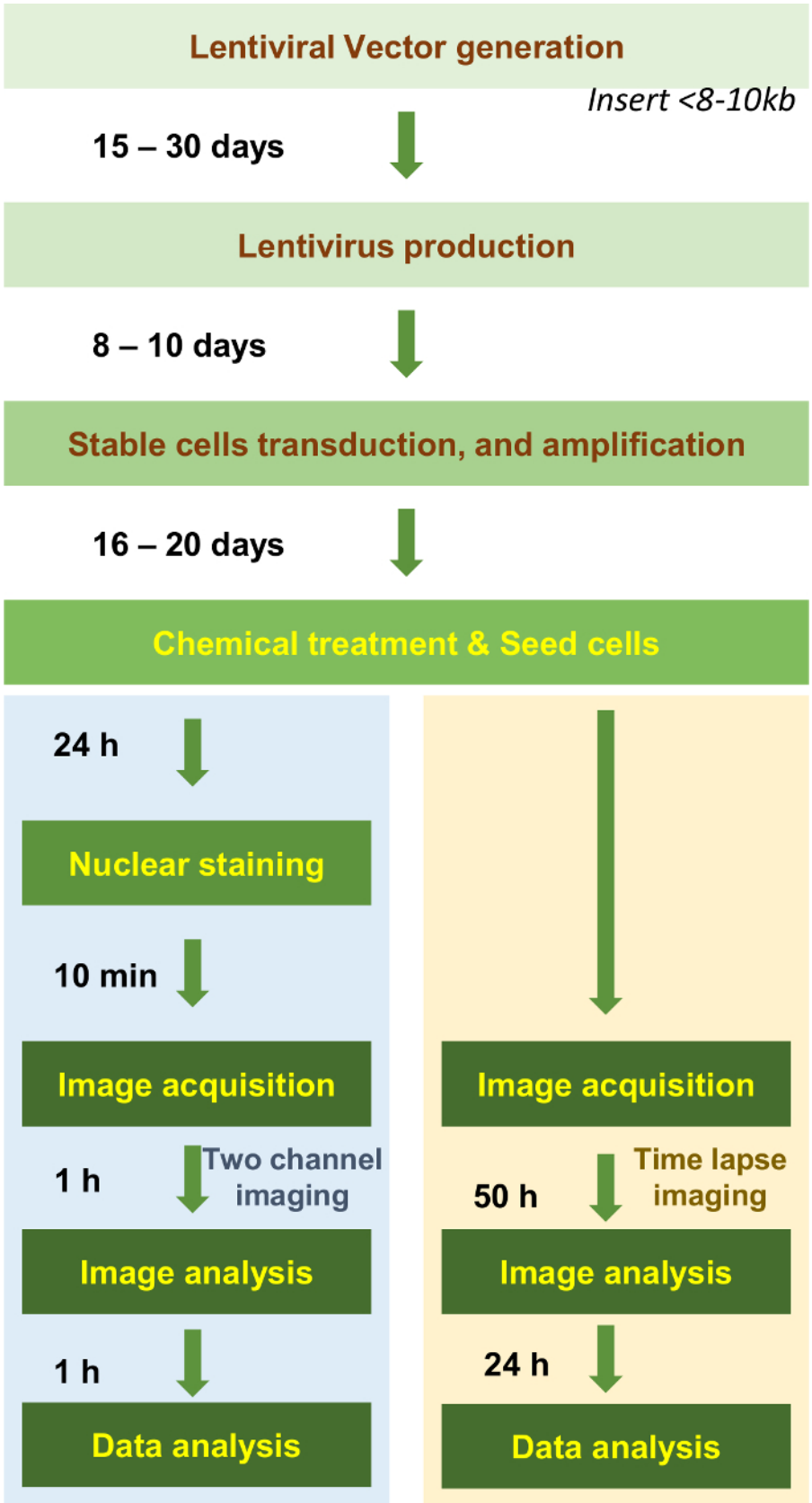

Figure 1. Workflow and timeline for cell-line generation and High content analysis (HCA) of protein aggregation. Please click here to view a larger version of this figure.

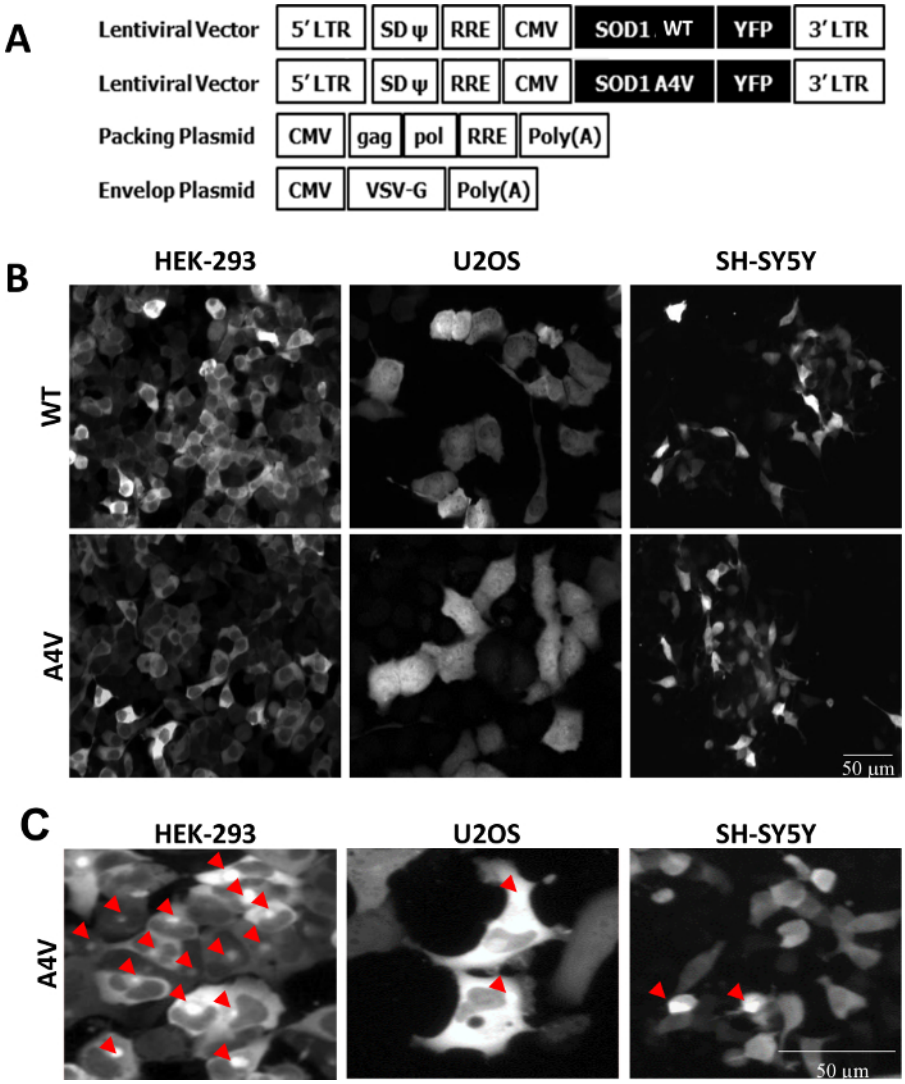

Figure 2. SOD1 WT and A4V stable line generation.
(A) Schematic diagram of lentiviral and packaging vectors (Packing and Envelop plasmids) for wild type and mutant SOD1 A4V lentivirus generation. **(B)** Selected confocal images of three cells (HEK-293, U2OS, and SH-SY5Y) transduced with the lentivirus wild type SOD1 (WT) and mutant SOD1 (A4V). **(C)** Representative images of three stable cells treated for 24 h with the proteasome inhibitor, ALLN (10µM). SOD1A4V-YFP aggregates were found to be abundantly present (red arrows) in HEK-293, but sparsely present in U2OS or SH-SY5Y cells. Images were acquired using a 20X objective. Scale bar = 50 µm. Please click here to view a larger version of this figure.

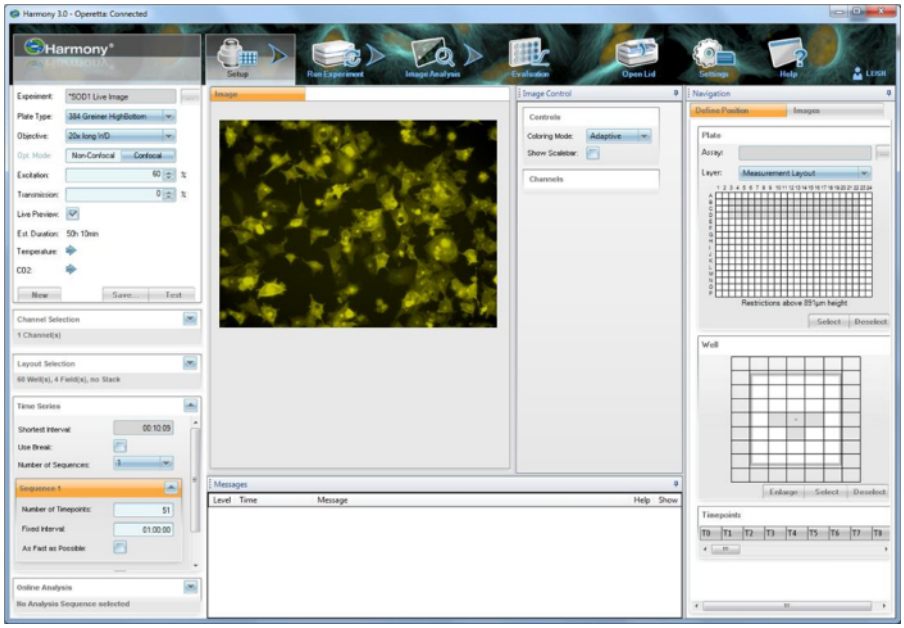

Figure 3. Screenshot of the experimental set up for a time-lapse using a microscope system with controlled environmental conditions (37 °C and 5% CO_2). Please click here to view a larger version of this figure.

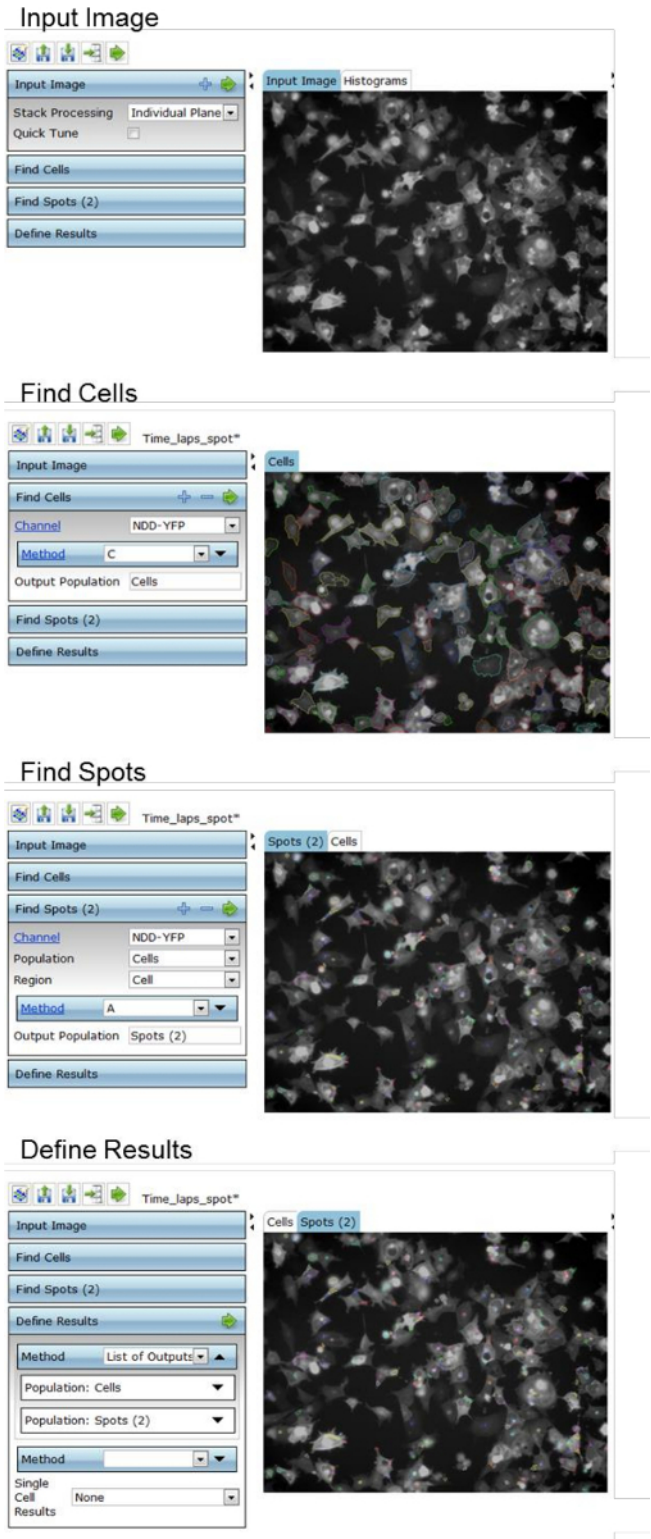

Figure 4. The four steps required for image analysis of aggregation using the YFP channel to detect cells and aggregates. (1) Import images from the image data storage and analysis system. (2) Select "find cells" for counting cells. (3) Select "find spots" to determine aggregates. (4) Define results based on each algorithm. Please click here to view a larger version of this figure.

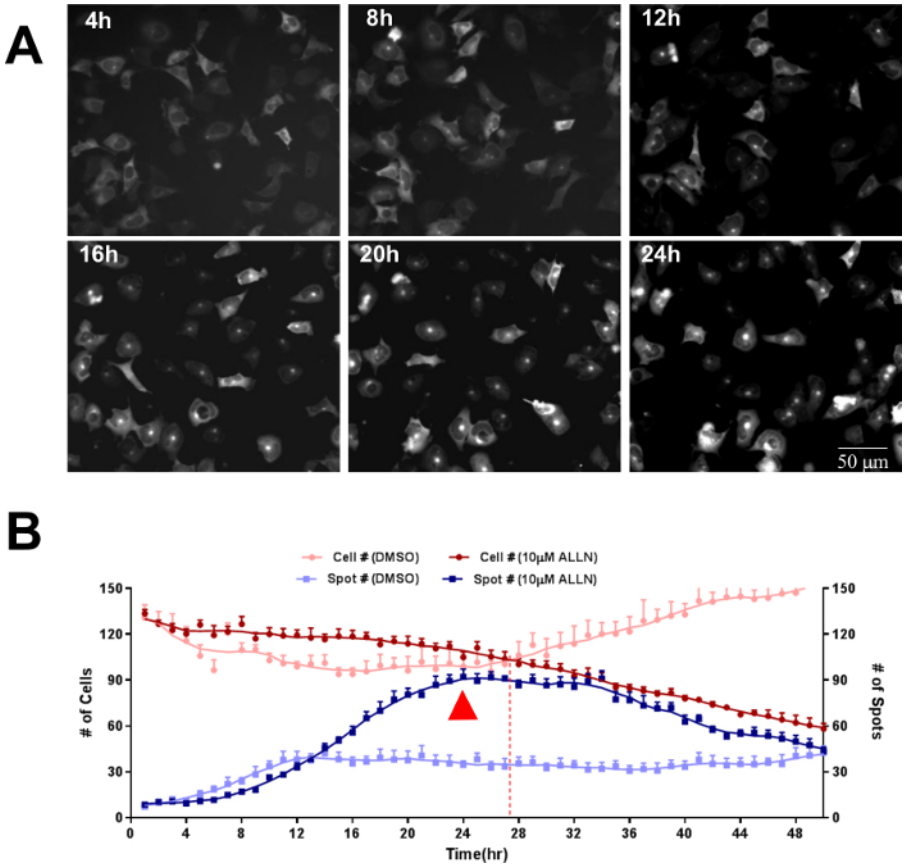

Figure 5. Proteasome inhibition leading to the accumulation of SOD1A4V-YFP aggregates in HEK-293 cells.
(A) Selected confocal images of SOD1A4V-YFP HEK-293 cells treated with 10 µM of ALLN. **(B)** Quantitative analysis of aggregate formation induced with ALLN (10µM) and cell counts in a time-dependent manner. Images were acquired using a LWD 20X objective every 60 min. Scale bar = 50 µm. Please click here to view a larger version of this figure.

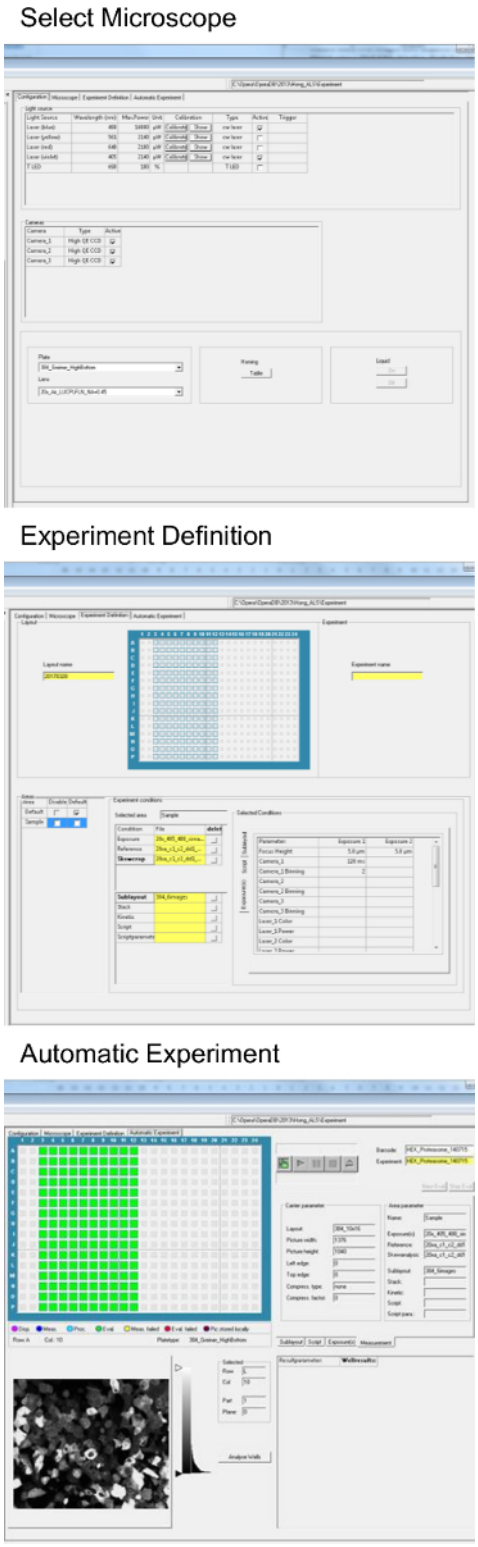

Figure 6. Screen shot of the experimental set up for two channel acquisition (YFP and Hoechst). Please click here to view a larger version of this figure.

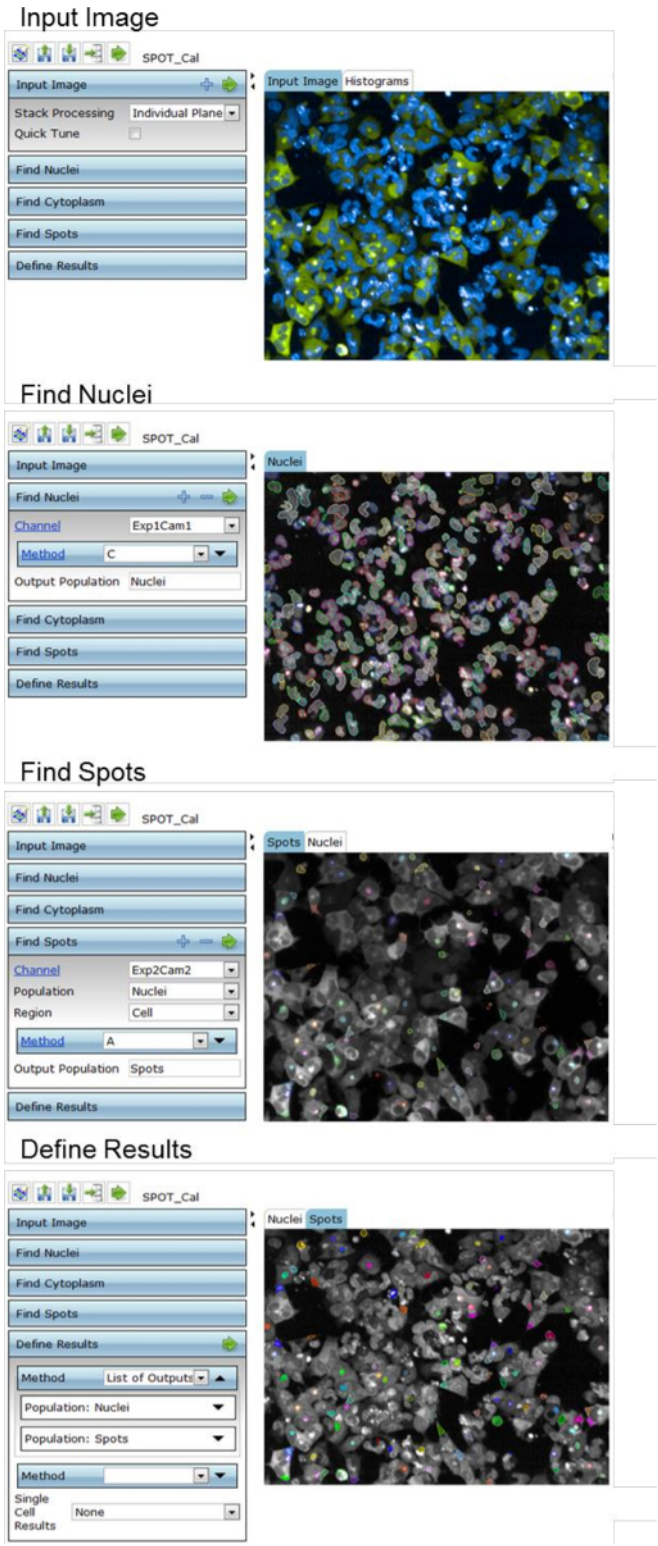

Figure 7. The four steps required for image analysis of SOD1 aggregates and cells labelled with YFP and Hoechst.
(1) Input images from the image data storage and analysis system. (2) Select "find nuclei" for counting cells. (3) Select "find spots" to determine aggregates. (4) Define results based on each algorithm. Please click here to view a larger version of this figure.

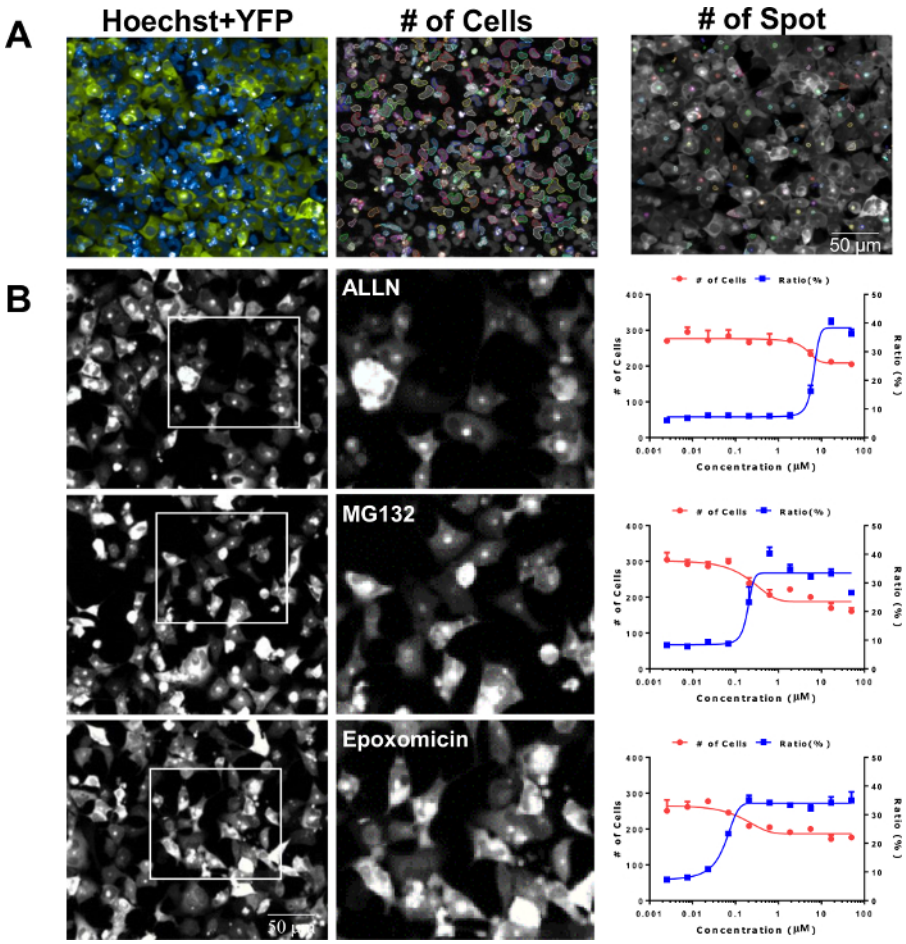

Figure 8. Quantitative analysis of SOD1 A4V aggregation and dose-dependent proteasome inhibitor concentration.
(A) Here we present the image and quantification analysis method for spots and cell counts using the Columbus image analysis system. Images corresponding to two fluorescent channels specific to Hoechst (Ch1) and YFP (Ch2) were sequentially acquired. Hoechst-stained objects (left) were identified by image segmentation. The nuclear mask (center) derived from the "find nuclei" algorithm was applied to count the number of cells, followed by the spot mask (right) "find spots" algorithm used to detect aggregates. **(B)** Dose response study of the proteasome inhibitors' effect on aggregation of mutant SOD1 A4V, which shows a variable EC_{50} ranking from 6.53 µM, ALLN, to 0.17 µM for MG132, and 0.03 µM for Expoxomicin. Selected confocal images of mutant SOD1 A4V treated proteasome at EC_{50} concentration. Scale bar = 50 µm. Please click here to view a larger version of this figure.

Discussion

There are two main approaches for generating stable cell lines. The first takes several weeks and requires transient transfection and resistance selection of the genomic integrated plasmid DNA vectors. The second takes a matter of hours through the use of lentivirus, making this protocol amenable to the effective expression of target protein in multiple cell lines with limited effort. The TRIP-CMV vector[19] was a sustained vector to use, with conserved transduction efficiency and stable transgene expression. To our surprise, the three cell lines generated showed no visible aggregation of the mutant SOD1, in contrast to the experimental protocol based on transient expression described earlier[11]. It is likely that transient expression favors protein aggregation as it produces higher protein expression in a relatively short period of time. Upon transient transfection, the ubiquitin–proteasome system, which degrades a majority of proteins, reaches a saturation point in which its capacity to degrade aggregate-prone proteins is fully utilized. However, we contend that stable lines are more likely to reproduce the physiological situation in which proteins are expressed at a relatively constant level which matches the elimination of misfolded protein via the proteasome, autophagosome-lysosome, and exocytosis. Although the primary events leading to ALS are still unknown, aging in connection with decreased activity of ubiquitin proteasome system, is a significant risk factor[23]. As illustrated by our cellular assay, proteasome inhibition promotes the aggregation of misfolded protein (SOD1 A4V). As such, this assay creates new opportunities to screen for small molecule activators of the autophagy signaling pathway or the chaperone network. Indeed, this avenue for chaperone induction seems promising, as illustrated by the report from Kieran *et al.* showing that the treatment with a co-inducer of the heat shock response was found to have therapeutic benefits by extending the lifespan of SODG93A mice[24].

It is important to mention that there are a few pitfalls to consider before using this assay. As with the assay optimization protocol, we found that concentrations of DMSO at 1% or above increases the mean intensity of the measured YFP in our assay. It is therefore important to reduce the concentration of DMSO to 0.5% to avoid any such artefact. Furthermore, the selection of the lens magnification and cell density in the assay plate are important to consider to optimize the robustness of the assay.

In our study, we have shown that using a lentiviral system, the SOD1A4V-YFP HEK-293 cell line is well suited for monitoring mutant SOD1 A4V aggregate formation upon induction by proteasome inhibitors. The study of misfolded protein and aggregation is essential for understanding the mechanisms of proteinopathies. Although the research tools for analyzing protein aggregation have been expanded in the past decades, efficient approaches to detect and quantify aggregation are required to screen molecules that prohibit aggregate formation. This work provides a detailed protocol for assay development and studies of protein aggregation. Hopefully, with the support of HCS, HCA and use of chemical or CRISPR/siRNA libraries, this work should open new avenues for therapeutics or novel target discovery.

Disclosures

The authors have nothing to disclose.

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