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Characterization of a Split-Nluc Assay for Monitoring Changes in Alpha-Synuclein Solubility in Living Cells

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Characterization of a Split-Nluc Assay for Monitoring Changes in Alpha-Synuclein Solubility in Living Cells

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Abstract

Alpha-synuclein is the primary constituent of Lewy Bodies, protein aggregates associated with Parkinson's Disease, Dementia with Lewy Bodies, and Multiple Systems Dystrophy. Given the terminal nature of these synucleinopathies, the need for an assay that can report on alpha-synuclein aggregation levels in living systems is critical. Western blotting represents one potential approach to this problem; however, this technique can often be limited by selectivity of available antibodies and experimental reproducibility. Herein, we describe a novel luminescence-based assay capable of detecting alpha-synuclein solubility in bacterial cells. This platform can report on the influence of disease-relevant mutations and small molecules. Antibodies are evaluated for use in Western blotting analysis to complement luminesce assay results. Preliminary data indicate that improved antibodies/Western blotting procedures are needed in order to visualize alpha-synuclein expression in this system. Thus, the luminescence-based assay for alpha-synuclein represents an attractive alternative for monitoring alpha-synuclein solubility and could ultimately be used to study the influence of mutations and tool compounds on alpha-synuclein solubility in living systems.

Key Words: Nanoluciferase, Protein Solubility Assay, Alpha-synuclein, Parkinson's Disease

Introduction

Parkinson's Disease and other Synucleinopathies

Parkinson's Disease (PD) is a motor system disorder predominantly characterized by symptoms such as tremors, rigid movements, bradykinesia, and postural inability. The disease primarily affects individuals 70 years and older, however symptoms can arise sooner in some cases.[1] PD currently affects 512 people out of 100,000 in the United States over the age of 45.[2] PD is a chronic progressive neurodegenerative disorder, meaning that its symptoms persist and become worse over an extended period of time due to its ability to reduce the function of neurons in the Central Nervous System. Brain cells responsible for the production of the neurotransmitter dopamine either become damaged or die. There is no known cure for the disease, and current treatment are aimed at alleviating symptoms. Early symptoms are treated with the drugs levodopa and carbidopa, though the efficacy of the drugs is reduced with time.[1] Surgery to implant microelectrodes for deep brain stimulation, diet, and rehabilitative exercises have been shown to improve symptoms.[3]

PD is one of many Synucleinopathies, a group of neurodegenerative diseases that are often terminal in nature. Synucleinopathies are characterized by mutations and post-translational modifications to the intrinsically disordered protein alpha-synuclein (aSYN) in select groups of neurons. aSYN is a protein found to be abundant in the human brain, often found at the pre-synaptic terminals of neurons. However, mutations and post-translational modifications cause aSYN to misfold, creating insoluble fibers that ultimately aggregate in association with vesicles and other components, termed Lewy Bodies (LBs) (Figure 1A-1C).[4] LBs have been shown to impede microtubule formation and cause mitochondrial and nuclear degradation, leading to loss

of function in the affected cells.[5] LB's also impede the delivery of dopamine to the substantia nigra, a portion of midbrain, causing cell death (Figure 1D).[6] Having a method for assessing aSYN aggregation in living systems could provide further insight into the underlying mechanisms of LB formation including compounds capable of modulating aSYN aggregation.[7]

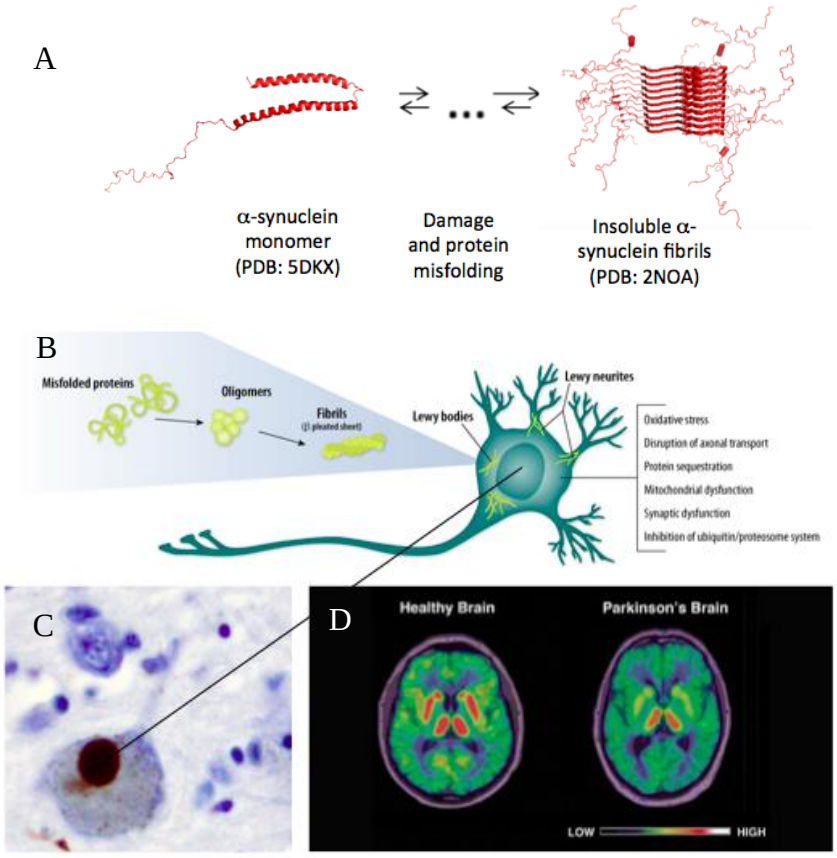


Figure 1 aSYN physiology. (A) aSYN is the primary component of Lewy bodies created as a result of conformational changes and misfolding mutations. (B) aSYN aggregation can be induced by oxidative stress, mitochondrial dysfunction, synaptic dysfunction, or inhibition of ubiquitin/proteasome systems. Misfolded aSYN aggregates into oligomers, fibrils, and eventually Lewy bodies that are the pathological hallmarks of Parkinson's disease. (C) Positive aSYN staining of a Lewy body from a Parkinson's disease patient. (D) In the healthy human brain, glucose consumption (red) is readily visible in the substantia nigra. This process is markedly absent in a brain affected with Parkinson's. Adapted from Jaiswal, S. et al, *Herbal. Med.* **1,** 1 (2015), Recchia, A. et al, *Neurobiol. Dis.* **30,** 8 (2008), and Spillantini, M. et al, *Nature.* **388,** 839 (1997).

Self-Assembling Split-Nanoluciferase Assay

Traditional methods for detecting aSYN aggregation involve the use of dyes, such Congo Red and Thioflavin T. However, these compounds only work *in vitro* rather than *in vivo* and are generally unable to report aggregation levels with high enough throughput for effective chemical, genetic, and chemical testing.[8] We have developed a novel luminescence-based assay monitoring the solubility of aSYN in bacterial cells. This assay relies on the spontaneous reassembly of NanoLuc luciferase fragments previously described by our lab.[9] NanoLuc is a recently developed small monomeric protein reporter that, when dissected into fragments, is capable of rapid reassembly and, in turn, production of a luminescent signal. In our assay, the N-terminus fragment consisting of residues 1-65 (N65) is fused with a protein of interest (POI). Rapid reassembly of N65 with the complementary fragment containing residues 66-171 (66C) leads to the formation of active NanoLuc and, in turn, a luminescent signal (Figure 2). The solubility of the POI influences the amount of N65 available for reassembly. Thus, luminescence signal from the sensor is directly proportional to the solubility of the POI.

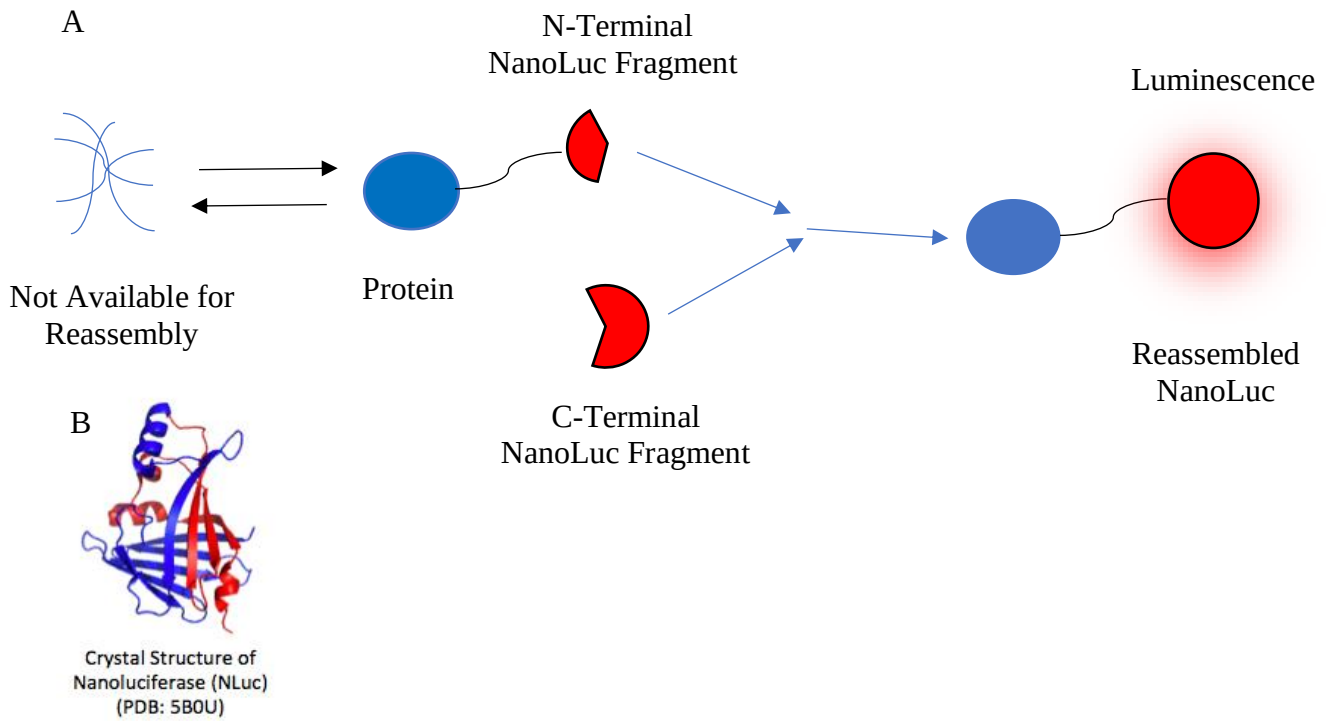


Figure 2. (A) Monitoring aSYN solubility using a split-NanoLuc assay. (A) Non-aggregated ASYN fused with N-terminus of Nanoluc spontaneously reassembles with the C-terminus of Nanoluc. Reassembled Nanoluc produces a luminescence signal that is proportional to the amount of aSYN. The solubility of a protein fused for the N-terminal NanoLuc fragment (N65) can be readout with the C-terminal Nluc fragment (66C) in living cells. (B) A crystal structure of Nluc. The N65/66C split halves are highlighted in red and blue, respectively.

Rationale

The assay shown in Figure 2A has been previously shown to report on the influence of mutations and small molecules on the solubility of amyloid-beta (Alzheimer's), huntingtin (Huntington's), and amylin (type 2 diabetes).[9] We have recently re-engineered this assay to report on changes in aSYN solubility in bacterial cells.[10] The goal of this project was to benchmark these efforts against Western blotting analysis of aSYN expression levels in our system, which can often be complicated by antibody selectivity and experimental reproducibility. Thus, we hypothesized that the split-Nluc assay for aSYN solubility may provide a more reproducible readout of aSYN solubility.

Comparing Western Blotting to the Luminescence Assay

Western Blotting is a technique used to selectively identify a POI from a mixture of proteins separate on an SDS-PAGE gel. The protein mixture is combined with a loading buffer such that it can be processed through SDS-PAGE gel. Use of SDS establishes a uniform negative on the proteins as well as denaturing the proteins (along with heating) such that separation in dependent upon primary sequence length. Betamercaptoethanol is also added in order to break up covalent, disulfide bonds.[11] The protein mixture can then be separate by size using electrophoresis (Figure 3).[11]

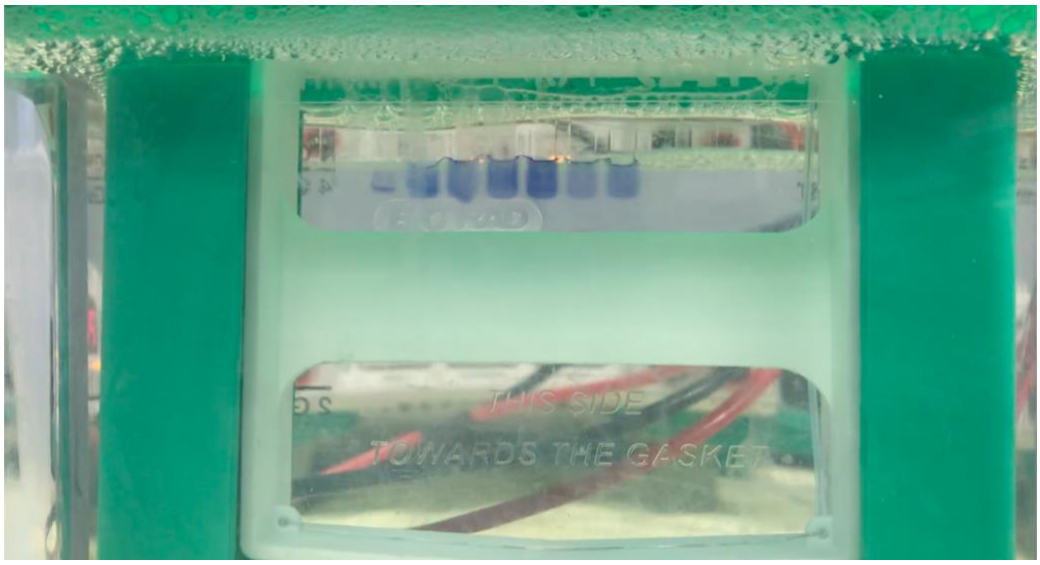


Figure 3. A typical SDS-PAGE gel. The protein mixture is allocated into wells present in the gel alongside a protein standard ladder for comparative reference. After a charge is applied, proteins migrate through the gel based on size.

After separation by SDS-PAGE , proteins are transferred to a nitrocellulose membrane, the membrane is “blocked” by washing it in nonfat dried milk diluted in 1x TBST for several hours, preventing nonspecific binding of antibodies to the nitrocellulose membrane. A primary specific to the POI is then incubated with the nitrocellulose membrane. Primary antibody binding is visualized using a and secondary antibody labeled with horseradish peroxidase allowing for subsequent imaging of the POI by chemiluminescence imaging. Comparison of the POI band to the ladder allows for relativistic determination of protein size in kilodaltons (kDa). If protein samples are normalized for protein content, relative POI band intensities can be compared. Clearly, this entire protocol is dependent upon the availability of selective antibodies for the POI as well as reproducibility of transfer, blocking, and incubation steps. We envisioned testing the reproducibility of Western blotting by growingBL21-Gold (Agilent, #230132) *E. coli* bacterial

cells expressing WT-aSYN-N65 and C66 in the absence or presence of known inhibitors of aSYN aggregation, EGCG[12] or D-Mannitol.[13] Western blotting results for changes in soluble aSYN would then be compared to previous luminescence results from the split-NanoLuc assay (Figure 4).

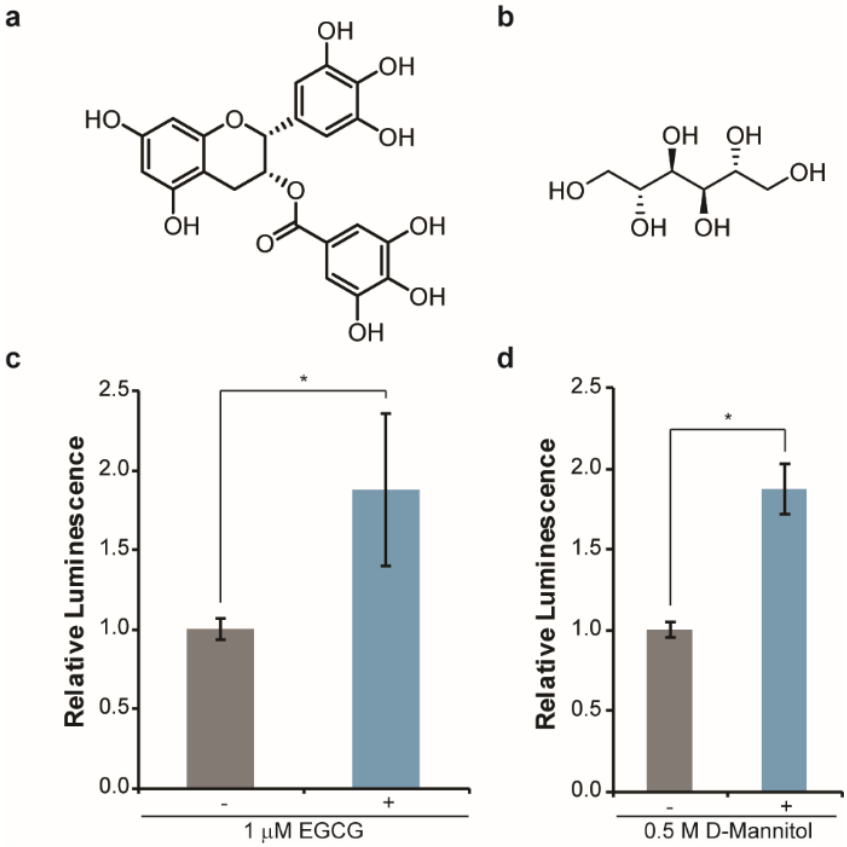


Figure 4. Observing aSYN solubility with known aggregation inhibitors. (a) EGCG structure (b) D-mannitol structure. Luminescence of bacterial cells expressing WT-αSYN-N65 and 66C in the presence or absence of the indicated concentration of EGCG (c) or D-mannitol (d). Error bars represent the standard deviation of three (EGCG) or two (D-Mannitol) biological replicates assayed in triplicate. * indicates a p-value of <0.05. Adapted from Nelson, T., Truong, T., Truong, B., Bilyeu, C., Zhao, J., and Stains, C. (2020).

Methods

D-Mannitol Media Preparation

Flasks containing TB liquid culture media were prepared according to manufacturer protocol. D-mannitol was added to the appropriate concentration (0.5M and 1.0 M). Alongside control TB, the TB with D-mannitol was autoclaved prior to use.

EGCG Media Preparation

EGCG (Cayman Chemical, 70935) was prepared at 100 mM in DMSO.

Cell Culture

BL21-Gold (Agilent, #230132) *E. coli* bacterial cells expressing WT-aSYN-N65 and 66C were grown in control TB (5mL) , TB with EGCG (5 mL), and TB with Mannitol (5mL) overnight with shaking at 37 ^{0}C in the presence of both ampicillin (100 μg/mL) and kanamycin (50 μg/mL). Optical densities were measured at 600 nm (OD_{600}), and the cultures were diluted to OD_{600} = 0.1 in the respective media. Cells were grown with shaking at 37 ^{0}C until an OD_{600} = 0.6-0.8 was achieved. IPTG (0.2 mM) and EGCG were induced afterwards, and the cells were incubated overnight at 16 ^{0}C. The following morning, the OD_{600} was measured and normalized to 3.0 in 1 mL. Cells were harvested at 700 g for 30 minutes at 4^{0}C then lysed with Bacteria Protein Extraction Reagent (B-PER, 30 μl, Thermo-Fischer). Cells were incubated for 10-15 minutes at room temperature, then centrifuged at 15,000 x g for 5 minutes.

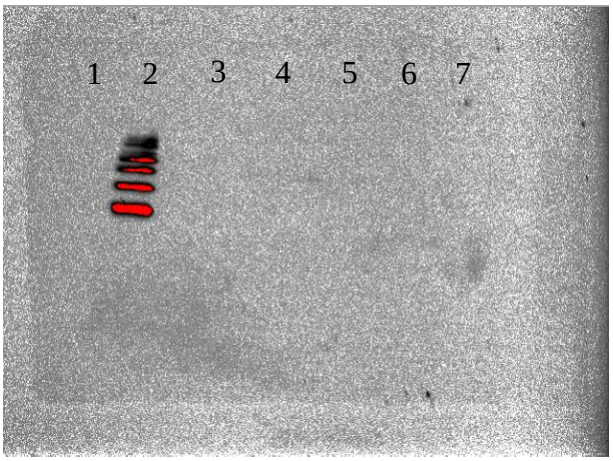
Western Blotting

Supernatant resulting from the centrifugation were mixed with 3 µl of SDS-PAGE loading dye (Glycerol, SDS, betamercaptoethanol, bromophenol blue, tris pH = 6.8) and denatured in boiling water (100^0C) for 10 minutes. Samples were centrifuged at 17,000 x g for 5 minutes and cooled to room temperature before being loading on a 12% SDS-PAGE gel alongside 2 ladders (Novex Sharp Protein Ladder (LC5801), Magic XP Western Ladder (LC5602) and running at 105 V for 90 minutes. Proteins were transferred onto a nitrocellulose membrane, using 100 V for 60 minutes. The membrane was blocked using nonfat milk (1g Dry Milk in 20 mL 1x TBST), then triple-washed with 1x TBST. The membrane was incubated with the primary antibody (alpha Synuclein Antibody (14H2L1), ABfinity™ Rabbit Monoclonal, 1:1,000) in 5 mL nonfat milk overnight at 4^0C. After primary antibody incubation, the membrane was triple-washed with 1x TBST and incubated with the secondary antibody (Goat, Anti-Rabbit Conjugated HRP, 1:1000) in nonfat milk for 60 minutes. The membrane was developed with Duraluminol (SuperSignal® West Pico Chemiluminescent Substrate) and imaged using a Gel Doc XR + System.

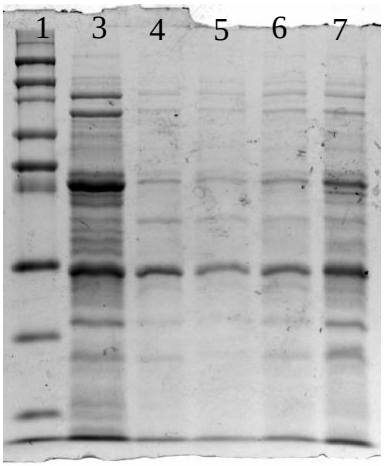
Results and Discussion

Unfortunately, we found that the antibody used in this work was not capable of detecting aSYN under the conditions described herein. Protein transfer was verified by chemiluminescence visualization of the Western blot standard (Figure 7A). Staining of the SDS-PAGE gel with Coomassie Brilliant Blue (R-250) dye revealed that protein was present in each lane, although normalization may not have been and should be optimized in future experiments(Figure 7B). Nonetheless, the absence of bands corresponding to aSYN in Western blots was highly reproducible under identical conditions with the aforementioned antibodies.

A



B



Lane	Sample Type
1	Novex Ladder
2	MagicMark Ladder
3	Control
4	0.5 M Mannitol
5	1 M Mannitol
6	10 nM EGCG
7	100 nM EGCG

Figure 7. Western blotting was not capable of detecting aSYN under the experimental conditions. (A) A Western blot of proteins isolated from BL-21 (Agilent, #230132) *E. coli* bacterial cells expressing WT-aSYN-N65 and C66 and was run at 105V for 90 minutes. Proteins were transferred to a nitrocellulose membrane at 100V for 60 minutes. 1_0 antibody: alpha Synuclein Antibody (14H2L1), ABfinity™ Rabbit Monoclonal, 1:1,000. 2_0 antibody: Goat, Anti-Rabbit Conjugated HRP, 1:1000. Only bands for the Western blot standard are observed in lane 2. (B) An SDS-PAGE gel stained with R-250 Coomassie Brilliant Blue dye prior to transfer to nitrocellulose. Proteins are presents in each lane.

Summary and Future Direction

In summary, a Western blotting under the experimental conditions described herein was found to be less reliable that the split-Nluc assay for monitoring changes in aSYN solubility. In the future, methods and protocols will need to be re-evaluated in order to potentially produce viable Western blotting results. Coomassie staining reveals that the proteins were successfully isolated from the cells of interest (Figure 7B), and the presence of a visible ladder on the Western Blot demonstrates that the proteins were successfully transferred to the membrane. Troubleshooting could involve screening additional primary antibodies as well as evaluating different cell extract preparations as previous has shown that aSYN may be expressed in the periplasm of bacteria.[17]

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