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## A luminescence-based assay for monitoring changes in alpha-synuclein aggregation in living cells†

Travis J. Nelson,<sup>‡,a</sup> Tiffany Truong,<sup>‡,§,a</sup> BaoLong Truong,<sup>a</sup> Camden V. Bilyeu,<sup>a</sup> Jia Zhao<sup>a</sup> and Cliff I. Stains  <sup>\*,abcde</sup>

Parkinson's disease is characterized by the accumulation of protein aggregates in the brain, termed Lewy bodies. Lewy bodies are predominantly composed of  $\alpha$ -synuclein and mutations that increase the aggregation potential of  $\alpha$ -synuclein have been associated with early on-set disease. Assays capable of reporting on the solubility of  $\alpha$ -synuclein in living cells could provide a means to interrogate the influence of mutations on aggregation as well as identify small molecules capable of modulating the aggregation of  $\alpha$ -synuclein. Herein, we repurpose our previously reported self-assembling NanoLuc luciferase fragments to engineer a platform for detecting  $\alpha$ -synuclein solubility in living cells. This new assay is capable of reporting on changes in  $\alpha$ -synuclein solubility caused by disease-relevant mutations as well as inhibitors of aggregation. In the long term, this new assay platform provides a means to investigate the influence of mutations on  $\alpha$ -synuclein solubility as well as identify potential tool compounds capable of modulating  $\alpha$ -synuclein aggregation.

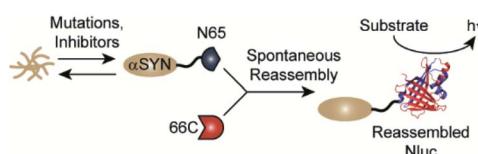
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Parkinson's disease (PD) is a major neurodegenerative disease, affecting an estimated 572 out of every 100 000 people in the US over the age of 45,<sup>1</sup> and is characterized by the formation of protein aggregates termed Lewy bodies (LBs). LBs consist primarily of  $\alpha$ -synuclein<sup>2,3</sup> and display a cross- $\beta$  structure.<sup>4</sup> Evidence indicates that LBs can be propagated in patients in a mechanism reminiscent of prion protein propagation.<sup>5</sup> Importantly, mutations that increase the aggregation potential of  $\alpha$ -synuclein ( $\alpha$ SYN) have been associated with more aggressive disease phenotypes.<sup>6</sup> Thus, methods for assessing  $\alpha$ SYN solubility in living cells could ultimately provide insights into mutant  $\alpha$ SYN aggregation potential as well as aid in the identification of compounds capable of modulating  $\alpha$ SYN aggregation.<sup>7,8</sup>

Classic methods for detection of protein aggregates rely on small molecule-based optical probes (such as thioflavin T and

Congo red).<sup>9–11</sup> While these methods remain the gold standards for *in vitro* experiments, recent efforts have focused on the development of approaches to assess protein aggregation within living systems.<sup>12–16</sup> Our lab has developed a cell-based, bioluminescent assay system that relies on the rapid reassembly of fragmented NanoLuc luciferase (Nluc),<sup>17</sup> an engineered luciferase that utilizes coelenterazine or analogues thereof as substrates.<sup>18–22</sup> In this system, proteins of interest (POIs) are fused to the N-terminus of residues 1–65 of Nluc (termed N65). Reassembly with the complementary Nluc fragment, consisting of residues 66–171 (termed 66C), leads to reassembly of active Nluc and production of a luminescent signal. Changes in the solubility of the POI influences the amount of N65 available for reassembly, making luminescence from this assay system proportional to the solubility of the POI (Fig. 1). We have previously shown that this assay platform is capable of



**Fig. 1** A split-Nluc assay system for interrogating changes in  $\alpha$ SYN solubility.  $\alpha$ SYN is covalently fused to the N-terminus of N65. Mutations to the  $\alpha$ SYN sequence or treatment with inhibitors alters the amount of  $\alpha$ SYN-N65 available for reassembly with 66C. Light production from reassembled Nluc (PDB: 5B0U) can be used as a proxy for soluble  $\alpha$ SYN.

<sup>a</sup>Department of Chemistry, University of Nebraska – Lincoln, Lincoln, NE 68588, USA.  
E-mail: cstains@virginia.edu

<sup>b</sup>Nebraska Center for Integrated Biomolecular Communication, University of Nebraska – Lincoln, Lincoln, NE 68588, USA

<sup>c</sup>Cancer Genes and Molecular Regulation Program, Fred & Pamela Buffet Cancer Center, University of Nebraska Medical Center, Omaha, NE 68198, USA

<sup>d</sup>Department of Chemistry, University of Virginia, Charlottesville, VA, 22904, USA

<sup>e</sup>University of Virginia Cancer Center, University of Virginia, Charlottesville, VA, 22904, USA

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‡ These authors contributed equally.

§ Present address: University of Nebraska Medical Center, Omaha, NE 68198, USA.



reporting the influence of mutations as well as small molecules on the solubility of amyloid-beta (Alzheimer's), huntingtin (Huntington's), and amylin (type 2 diabetes).<sup>23,24</sup> Herein, we re-engineer this system to report on the solubility of  $\alpha$ SYN in living cells. We demonstrate the ability to detect the influence of disease-relevant mutations and small molecules on the solubility of  $\alpha$ SYN. In the long term, this new assay platform could be used to study the influence of mutations on aggregation in living cells as well as identify tool compounds capable of modulating aggregation.<sup>25–28</sup>

In order to engineer a split-Nluc system for detection of  $\alpha$ SYN solubility, we fused wild-type (WT)- $\alpha$ SYN to the N-terminus of N65. We then asked if this new assay platform was capable of reporting on the influence of mutations on  $\alpha$ SYN solubility. Specifically, we chose the A30P<sup>6,29–31</sup> and A53T<sup>32</sup> mutations that have been shown to increase oligomerization of  $\alpha$ SYN. In addition, we chose to investigate the influence of the G51D mutation that has been shown to decrease the rate of aggregation of  $\alpha$ SYN.<sup>33</sup> Accordingly, we performed site-directed mutagenesis to generate these mutants (Fig. 2a and Table S1†). Bacterial cells were transformed with each N65 fusion along with 66C.<sup>17</sup> Expression was induced with IPTG, cultures were normalized for cell density, and luminescence from reassembled Nluc was determined. We observed 89% and 61% decreases in the luminescence of cells expressing the A30P and A53T mutants relative to WT- $\alpha$ SYN (Fig. 2b), consistent with previous reports.<sup>6,29–32</sup> Interestingly, we note that previous work indicates that both A30P and A53T have a higher propensity to form oligomers *in vitro* as well as in cell-based assays.<sup>32,34</sup> In order to further probe the ability of our system to detect  $\alpha$ SYN oligomerization, we turned our attention to the disease-associated H50Q and E46K mutants that have been shown to display similar oligomer formation compared to WT- $\alpha$ SYN *in*

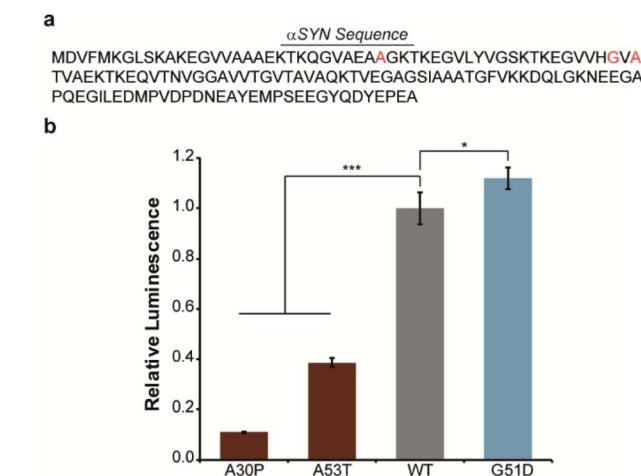


Fig. 2 Disease-relevant mutations influence  $\alpha$ SYN solubility. (a) The amino acid sequence of  $\alpha$ SYN is shown with mutation sites indicated in red. (b) Luminescence from bacterial cells expressing the indicated mutant or wild-type (WT)  $\alpha$ SYN fused to N65 in the presence of 66C. Error bars represent the standard deviation of two (A30P) or three (A53T, WT, and G51D) biological replicates assayed in triplicate. \* indicates a  $p$ -value of  $<0.05$  and \*\*\* indicates a  $p$ -value of  $<0.001$ .

*vitro* and in mammalian cells.<sup>34–36</sup> As expected, the H50Q and E46K mutants display indistinguishable luminescent intensities in the assay compared to WT- $\alpha$ SYN (Fig. S1†). In addition, we assessed the influence of the synthetic A30P/A76P mutant, which has been shown to increase oligomerization.<sup>34,37</sup> The A30P/A76P mutant displayed a significant 77% reduction in luminescence relative to WT- $\alpha$ SYN (Fig. S1†). Consequently, the split-Nluc system may be reporting on differences in the oligomer formation of these mutants relative to WT- $\alpha$ SYN, this observation is being further investigated by our laboratory. Conversely, the G51D mutant displayed a reproducible 12% increase in luminescence, in-line with previous observations of reduced aggregation kinetics of this mutant.<sup>33</sup> Regardless of the specific identity of the aggregate species that inhibits reassembly of the split-Nluc assay system, this platform is clearly capable of reporting on established trends in  $\alpha$ SYN aggregation as a result of mutations.

Next, we asked whether this re-engineered system was capable of reporting on the influence of small molecules on  $\alpha$ SYN solubility. For validation purposes, we chose molecules that have been previously shown to disrupt  $\alpha$ SYN aggregation both *in vitro* and in cells, namely EGCG<sup>38,39</sup> and  $\alpha$ -mannitol (Fig. 3a and b).<sup>40,41</sup> Accordingly, bacteria expressing WT- $\alpha$ SYN-N65 and 66C were grown in the presence of these inhibitors, normalized to cell density, and assayed for luminescence. We observed a reproducible 88% increase in luminescence from cells cultured in the presence of 1  $\mu$ M EGCG (Fig. 3c). Increasing the concentration of EGCG to 10  $\mu$ M did not result in

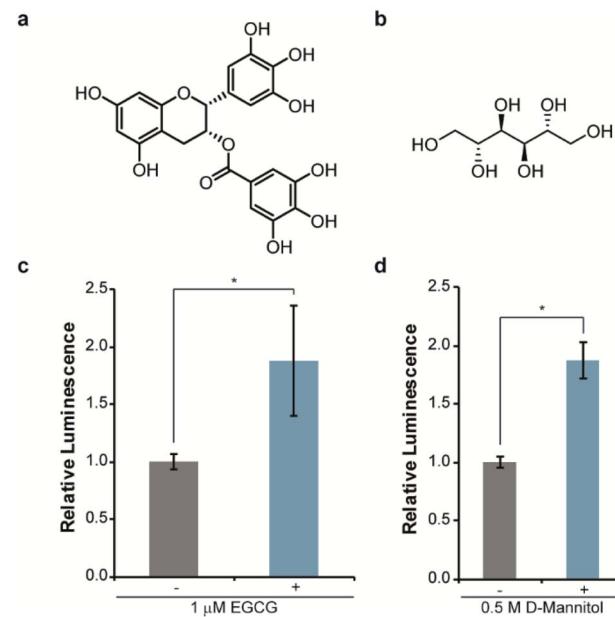


Fig. 3 Monitoring changes in  $\alpha$ SYN solubility upon treatment with known inhibitors of aggregation. The structures of EGCG (a) and  $\alpha$ -mannitol (b) are shown. Luminescence from bacterial cells expressing WT- $\alpha$ SYN-N65 and 66C in the absence or presence of the indicated concentration of EGCG (c) or  $\alpha$ -mannitol (d). Error bars represent the standard deviation of three (EGCG) or two ( $\alpha$ -mannitol) biological replicates assayed in triplicate. \* indicates a  $p$ -value of  $<0.05$ .



significantly increased luminescent signal (Fig. S2†) compared to 1  $\mu$ M EGCG (Fig. 3c), indicating that EGCG was saturating under these experimental conditions. Likewise, we observed an 88% increase in luminescence from cells treated with 0.5 M D-mannitol (Fig. 3d). In agreement with previous literature, increasing the concentration of D-mannitol to 1 M resulted in inhibition of cell growth and a decrease in inhibitory activity as assessed by luminescence (Fig. S3†). This data supports the observation of a reversed dose-dependent response for D-mannitol.<sup>40</sup> Taken together, these experiments demonstrate the ability of the split-Nluc system to report upon changes in  $\alpha$ SYN solubility in response to known inhibitors of  $\alpha$ SYN aggregation.

## Conclusions

We have re-engineered our split-Nluc assay system to provide a platform for the analysis of  $\alpha$ SYN solubility in cells. This method provides a luminescent readout of  $\alpha$ SYN solubility and is capable of reporting on the influence of mutations as well as small molecules on the aggregation of  $\alpha$ SYN. We note that previous work indicates that  $\alpha$ SYN is translocated to the periplasm of *E. coli*.<sup>42</sup> Ongoing work in our lab is aimed at determining whether the split-Nluc system described herein detects periplasmic and/or cytosolic populations of  $\alpha$ SYN. Nonetheless, the ability to detect previously reported changes in  $\alpha$ SYN solubility in response to six different point mutations suggests that the system is reporting on changes in solubility. Moreover, treatment with two small molecule inhibitors known to increase the solubility of  $\alpha$ SYN clearly results in an increase in luminescence of the assay system. Thus, while we cannot rule out changes in translocation influencing luminescence in all cases, the effects observed herein are attributed primarily to previously reported changes in  $\alpha$ SYN solubility. In the long term, we envision that this approach could be utilized to probe fundamental aspects of  $\alpha$ SYN folding as well as identify tool compounds capable modulating the aggregation of  $\alpha$ SYN within cells.

## Conflicts of interest

There are no conflicts to declare.

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