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

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Parkinson's disease is characterized by the accumulation of protein aggregates in the brain, termed Lewy bodies. Lewy bodies are predominantly composed of α-synuclein and mutations that increase the aggregation potential of α-synuclein have been associated with early on-set disease. Assays capable of reporting on the solubility of α-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 α-synuclein. Herein, we repurpose our previously reported self-assembling NanoLuc luciferase fragments to engineer a platform for detecting α-synuclein solubility in living cells. This new assay is capable of reporting on changes in α-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 α-synuclein solubility as well as identify potential tool compounds capable of modulating α-synuclein aggregation.

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,[1] and is characterized by the formation of protein aggregates termed Lewy bodies (LBs). LBs consist primarily of α-synuclein[2,3] and display a cross-β structure.[4] Evidence indicates that LBs can be propagated in patients in a mechanism reminiscent of prion protein propagation.[5] Importantly, mutations that increase the aggregation potential of α-synuclein (αSYN) have been associated with more aggressive disease phenotypes.[6] Thus, methods for assessing αSYN solubility in living cells could ultimately provide insights into mutant αSYN aggregation potential as well as aid in the identification of compounds capable of modulating αSYN aggregation.[7,8]

Classic methods for detection of protein aggregates rely on small molecule-based optical probes (such as thioflavin T and Congo red).[9–11] 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.[12–16] Our lab has developed a cell-based, bioluminescent assay system that relies on the rapid reassembly of fragmented NanoLuc luciferase (Nluc),[17] an engineered luciferase that utilizes coelenterazine or analogues thereof as substrates.[18–22] 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

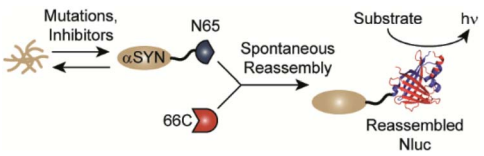

Fig. 1 A split-Nluc assay system for interrogating changes in αSYN solubility. αSYN is covalently fused to the N-terminus of N65. Mutations to the αSYN sequence or treatment with inhibitors alters the amount of αSYN-N65 available for reassembly with 66C. Light production from reassembled Nluc (PDB: 5B0U)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

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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).[23,24] Herein, we re-engineer this system to report on the solubility of αSYN in living cells. We demonstrate the ability to detect the influence of disease-relevant mutations and small molecules on the solubility of αSYN. 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.[25–28]

In order to engineer a split-Nluc system for detection of αSYN solubility, we fused wild-type (WT)-αSYN to the N-terminus of N65. We then asked if this new assay platform was capable of reporting on the influence of mutations on αSYN solubility. Specifically, we chose the A30P[6,29–31] and A53T[32] mutations that have been shown to increase oligomerization of αSYN. In addition, we chose to investigate the influence of the G51D mutation that has been shown to decrease the rate of aggregation of αSYN.[33] 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.[17] 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-αSYN (Fig. 2b), consistent with previous reports.[6,29–32] 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.[32,34] In order to further probe the ability of our system to detect αSYN 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-αSYN *in vitro* and in mammalian cells.[34–36] As expected, the H50Q and E46K mutants display indistinguishable luminescent intensities in the assay compared to WT-αSYN (Fig. S1†). In addition, we assessed the influence of the synthetic A30P/A76P mutant, which has been shown to increase oligomerization.[34,37] The A30P/A76P mutant displayed a significant 77% reduction in luminescence relative to WT-αSYN (Fig. S1†). Consequently, the split-Nluc system may be reporting on differences in the oligomer formation of these mutants relative to WT-αSYN, 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.[33] 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 αSYN 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 αSYN solubility. For validation purposes, we chose molecules that have been previously shown to disrupt αSYN aggregation both *in vitro* and in cells, namely EGCG[38,39] and D-mannitol (Fig. 3a and b).[40,41] Accordingly, bacteria expressing WT-αSYN-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 μM EGCG (Fig. 3c). Increasing the concentration of EGCG to 10 μM did not result in

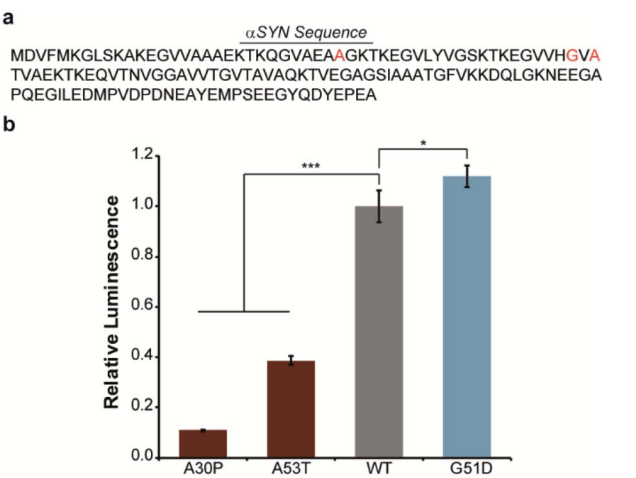

Fig. 2 Disease-relevant mutations influence αSYN solubility. (a) The amino acid sequence of αSYN is shown with mutation sites indicated in red. (b) Luminescence from bacterial cells expressing the indicated mutant or wild-type (WT) αSYN fused to N65 in the presence of 66C. Error bars represent the standard 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.

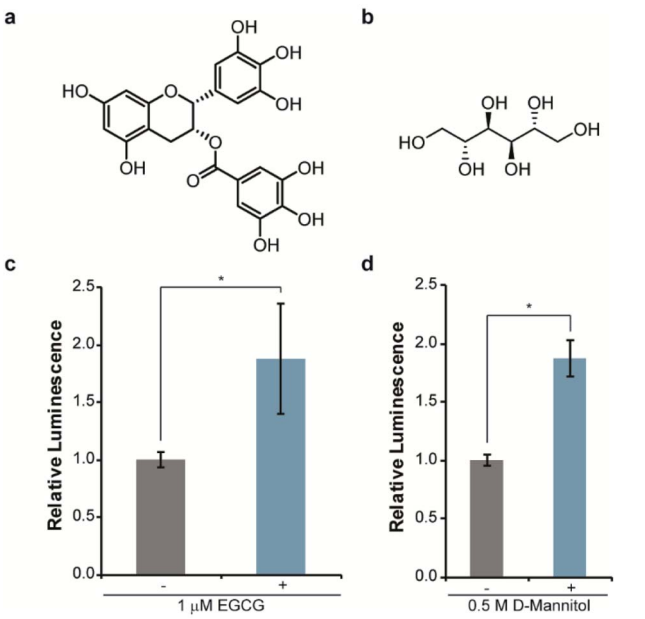

Fig. 3 Monitoring changes in αSYN solubility upon treatment with known inhibitors of aggregation. The structures of EGCG (a) and D-mannitol (b) are shown. Luminescence from bacterial cells expressing WT-αSYN-N65 and 66C in the absence of presence of the indicated concentration of EGCG (c) or D-mannitol (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.

significantly increased luminescent signal (Fig. S2†) compared to 1 μM 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.[40] Taken together, these experiments demonstrate the ability of the split-Nluc system to report upon changes in αSYN solubility in response to known inhibitors of αSYN aggregation.

Conclusions

We have re-engineered our split-Nluc assay system to provide a platform for the analysis of αSYN solubility in cells. This method provides a luminescent readout of αSYN solubility and is capable of reporting on the influence of mutations as well as small molecules on the aggregation of αSYN. We note that previous work indicates that αSYN is translocated to the periplasm of *E. coli*.[42] Ongoing work in our lab is aimed at determining whether the split-Nluc system described herein detects periplasmic and/or cytosolic populations of αSYN. Nonetheless, the ability to detect previously reported changes in αSYN 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 αSYN 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 αSYN solubility. In the long term, we envision that this approach could be utilized to probe fundamental aspects of αSYN folding as well as identify tool compounds capable modulating the aggregation of αSYN within cells.

Conflicts of interest

There are no conflicts to declare.

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References

1 C. Marras, J. C. Beck, J. H. Bower, E. Roberts, B. Ritz, G. W. Ross, R. D. Abbott, R. Savica, S. K. Van Den Eeden, A. W. Willis, C. M. Tanner and P. G. Parkinson's Foundation, *npj Parkinson's Dis.*, 2018, **4**, 21.
2 Q. Xia, L. Liao, D. Cheng, D. M. Duong, M. Gearing, J. J. Lah, A. I. Levey and J. Peng, *Front. Biosci.*, 2008, **13**, 3850–3856.
3 M. G. Spillantini, M. L. Schmidt, V. M. Lee, J. Q. Trojanowski, R. Jakes and M. Goedert, *Nature*, 1997, **388**, 839–840.
4 M. D. Tuttle, G. Comellas, A. J. Nieuwkoop, D. J. Covell, D. A. Berthold, K. D. Kloepper, J. M. Courtney, J. K. Kim, A. M. Barclay, A. Kendall, W. Wan, G. Stubbs, C. D. Schwieters, V. M. Lee, J. M. George and C. M. Rienstra, *Nat. Struct. Mol. Biol.*, 2016, **23**, 409–415.
5 K. Araki, N. Yagi, K. Aoyama, C. J. Choong, H. Hayakawa, H. Fujimura, Y. Nagai, Y. Goto and H. Mochizuki, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 17963–17969.
6 L. Narhi, S. J. Wood, S. Steavenson, Y. Jiang, G. M. Wu, D. Anafi, S. A. Kaufman, F. Martin, K. Sitney, P. Denis, J. C. Louis, J. Wypych, A. L. Biere and M. Citron, *J. Biol. Chem.*, 1999, **274**, 9843–9846.
7 J. A. Kritzer, S. Hamamichi, J. M. McCaffery, S. Santagata, T. A. Naumann, K. A. Caldwell, G. A. Caldwell and S. Lindquist, *Nat. Chem. Biol.*, 2009, **5**, 655–663.
8 T. F. Outeiro and S. Lindquist, *Science*, 2003, **302**, 1772–1775.
9 M. Biancalana and S. Koide, *Biochim. Biophys. Acta*, 2010, **1804**, 1405–1412.
10 H. Puchtler and F. Sweat, *J. Histochem. Cytochem.*, 1965, **13**, 693–694.
11 H. Puchtler, F. Sweat and M. Levine, *J. Histochem. Cytochem.*, 1962, **10**, 355–364.
12 K. L. Maxwell, A. K. Mittermaier, J. D. Forman-Kay and A. R. Davidson, *Protein Sci.*, 1999, **8**, 1908–1911.
13 G. S. Waldo, B. M. Standish, J. Berendzen and T. C. Terwilliger, *Nat. Biotechnol.*, 1999, **17**, 691–695.
14 W. C. Wigley, R. D. Stidham, N. M. Smith, J. F. Hunt and P. J. Thomas, *Nat. Biotechnol.*, 2001, **19**, 131–136.
15 Y. Liu, M. Fares, N. P. Dunham, Z. Gao, K. Miao, X. Jiang, S. S. Bollinger, A. K. Boal and X. Zhang, *Angew. Chem., Int. Ed.*, 2017, **56**, 8672–8676.
16 K. H. Jung, S. F. Kim, Y. Liu and X. Zhang, *ChemBioChem*, 2019, **20**, 1078–1087.
17 T. J. Nelson, J. Zhao and C. I. Stains, *Methods Enzymol.*, 2019, **622**, 55–66.
18 A. S. Dixon, M. K. Schwinn, M. P. Hall, K. Zimmerman, P. Otto, T. H. Lubben, B. L. Butler, B. F. Binkowski, T. Machleidt, T. A. Kirkland, M. G. Wood, C. T. Eggers, L. P. Encell and K. V. Wood, *ACS Chem. Biol.*, 2016, **11**, 400–408.
19 M. P. Hall, J. Unch, B. F. Binkowski, M. P. Valley, B. L. Butler, M. G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T. Machleidt, M. B. Robers, H. A. Benink, C. T. Eggers, M. R. Slater, P. L. Meisenheimer, D. H. Klaubert, F. Fan, L. P. Encell and K. V. Wood, *ACS Chem. Biol.*, 2012, **7**, 1848–1857.
20 O. Shimomura, T. Masugi, F. H. Johnson and Y. Haneda, *Biochemistry*, 1978, **17**, 994–998.
21 Y. Tomabechi, T. Hosoya, H. Ehara, S. I. Sekine, M. Shirouzu and S. Inouye, *Biochem. Biophys. Res. Commun.*, 2016, **470**, 88–93.
22 H. W. Yeh, O. Karmach, A. Ji, D. Carter, M. M. Martins-Green and H. W. Ai, *Nat. Methods*, 2017, **14**, 971–974.
23 J. Zhao, T. J. Nelson, Q. Vu, T. Truong and C. I. Stains, *ACS Chem. Biol.*, 2016, **11**, 132–138.

24 J. Zhao, Q. Vu and C. I. Stains, *Mol. BioSyst.*, 2016, **12**, 2984–2987.
25 W. Kim and M. H. Hecht, *J. Mol. Biol.*, 2008, **377**, 565–574.
26 A. F. McKoy, J. Chen, T. Schupbach and M. H. Hecht, *J. Biol. Chem.*, 2012, **287**, 38992–39000.
27 C. I. Stains, K. Mondal and I. Ghosh, *ChemMedChem*, 2007, **2**, 1674–1692.
28 C. Wurth, N. K. Guimard and M. H. Hecht, *J. Mol. Biol.*, 2002, **319**, 1279–1290.
29 H. A. Lashuel, B. M. Petre, J. Wall, M. Simon, R. J. Nowak, T. Walz and P. T. Lansbury, Jr, *J. Mol. Biol.*, 2002, **322**, 1089–1102.
30 J. Li, V. N. Uversky and A. L. Fink, *Biochemistry*, 2001, **40**, 11604–11613.
31 J. Li, V. N. Uversky and A. L. Fink, *Neurotoxicology*, 2002, **23**, 553–567.
32 K. A. Conway, S. J. Lee, J. C. Rochet, T. T. Ding, R. E. Williamson and P. T. Lansbury, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 571–576.
33 M. B. Fares, N. Ait-Bouziad, I. Dikiy, M. K. Mbefo, A. Jovicic, A. Kiely, J. L. Holton, S. J. Lee, A. D. Gitler, D. Eliezer and H. A. Lashuel, *Hum. Mol. Genet.*, 2014, **23**, 4491–4509.
34 D. F. Lazaro, E. F. Rodrigues, R. Langohr, H. Shahpasandzadeh, T. Ribeiro, P. Guerreiro, E. Gerhardt, K. Krohnert, J. Klucken, M. D. Pereira, B. Popova, N. Kruse, B. Mollenhauer, S. O. Rizzoli, G. H. Braus, K. M. Danzer and T. F. Outeiro, *PLoS Genet.*, 2014, **10**, e1004741.
35 D. Ghosh, M. Mondal, G. M. Mohite, P. K. Singh, P. Ranjan, A. Anoop, S. Ghosh, N. N. Jha, A. Kumar and S. K. Maji, *Biochemistry*, 2013, **52**, 6925–6927.
36 R. A. Fredenburg, C. Rospigliosi, R. K. Meray, J. C. Kessler, H. A. Lashuel, D. Eliezer and P. T. Lansbury, Jr, *Biochemistry*, 2007, **46**, 7107–7118.
37 D. P. Karpinar, M. B. Balija, S. Kugler, F. Opazo, N. Rezaei-Ghaleh, N. Wender, H. Y. Kim, G. Taschenberger, B. H. Falkenburger, H. Heise, A. Kumar, D. Riedel, L. Fichtner, A. Voigt, G. H. Braus, K. Giller, S. Becker, A. Herzig, M. Baldus, H. Jackle, S. Eimer, J. B. Schulz, C. Griesinger and M. Zweckstetter, *EMBO J.*, 2009, **28**, 3256–3268.
38 J. Bieschke, J. Russ, R. P. Friedrich, D. E. Ehrnhoefer, H. Wobst, K. Neugebauer and E. E. Wanker, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 7710–7715.
39 D. E. Ehrnhoefer, J. Bieschke, A. Boeddrich, M. Herbst, L. Masino, R. Lurz, S. Engemann, A. Pastore and E. E. Wanker, *Nat. Struct. Mol. Biol.*, 2008, **15**, 558–566.
40 R. Shaltiel-Karyo, M. Frenkel-Pinter, E. Rockenstein, C. Patrick, M. Levy-Sakin, A. Schiller, N. Egoz-Matia, E. Masliah, D. Segal and E. Gazit, *J. Biol. Chem.*, 2013, **288**, 17579–17588.
41 A. Paul, B. D. Zhang, S. Mohapatra, G. Li, Y. M. Li, E. Gazit and D. Segal, *Front. Mol. Biosci.*, 2019, **6**, 16.
42 G. Ren, X. Wang, S. Hao, H. Hu and C. C. Wang, *J. Bacteriol.*, 2007, **189**, 2777–2786.