COMPARISON OF ALPHA-SYNUCLEIN AGGREGATION KINETICS IN THE PRESENCE OF LIMP-2 OVEREXPRESSION IN PARKINSONS DISEASE MODEL.

By

Meshal Aldhubayb

B.S. (Majmaah University) 2014

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

September 2018

Accepted:

_____	_____
Ricky Hirschhorn, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
_____	
Craig Laufer, Ph.D. Committee Member	
_____	_____
Rachel Beyer, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

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DEDICATION

I dedicate this project to my father and mother who strongly support me even though I am thousand miles away from them and I would like to dedicate this project to my brothers and sisters. Last but not least, I dedicate this work to my friends in Saudi Arabia and in the United States.

ACKNOWLEDGEMENTS

I am grateful to everyone that helped me to develop this proposal. I would like to express my exceptional gratitude to Prof. Beyer who advise me in every particular point in my project. She spent a lot of time and effort in assisting and guiding me which without her nothing would be done. Furthermore, I would like to thank Prof. Hirschhorn who has supported and taught me during my study. Also, I would like to thank Prof. Boyd and Prof. Laufer who excellently taught me what I need to learn in the molecular biology field.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*

COMPARISON OF ALPHA-SYNUCLEIN AGGREGATION KINETICS IN THE PRESENCE OF LIMP-2 OVEREXPRESSION IN PARKINSONS DISEASE MODEL.

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
Aldhubayb, Meshal, Mohammed A	B.A.	N/A

3c. POSITION TITLE
Graduate Student

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Frederick, MD 21201

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Biology

3f. MAJOR SUBDIVISION
Biomedical Science Program

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: N/A FAX: N/A

E-MAIL ADDRESS:

4. HUMAN SUBJECTS RESEARCH ☐ No ☒ Yes
4a. Research Exempt: N/A

4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
N/A	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes
5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)

9. APPLICANT ORGANIZATION
Name: Meshal Aldhubayb
Address: Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.	SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)* N/A	DATE

Program Director/Principal Investigator (Last, First): Last Name, First Name

PROJECT SUMMARY (See instructions):

Parkinson's disease is a slow aggressive neurological disorder characterized by loss of dopamine in substantia nigra which is located in the brain and formats a neuronal cytotoxic protein called alpha- synuclein. Researchers found that genetics plays a significant role in formation of alpha-synuclein aggregation. Studies of families with the history of Parkinson's disease have identificatied of familial mutations (A30P, E46K, H50Q, G51D, A53T). Researchers have tried to prevent alpha-synuclein aggregation as a way to treat Parkinson's disease. One possible and promising way to prevent aggregation is overexpression of the lysosomal Integral Membrane Protein type-2 (LIMP-2). In this project, I propose to investigate the role of LIMP-2 in the familial mutation of Parkinson's diseases. This project uses cellular models for each of the familial mutations for Parkinson's diseases, inserts of plasmids that contain the LIMP-2 to these cellular models to investigate the effect of the LIMP-2 added on proved aggregation in the familial mutations, and monitor alpha-synuclein aggregation by using Fluorescence Resonance Energy Transfer (FRET) to measure it.

RELEVANCE (See instructions):

In this study, we investigate the role of LIMP-2 in decreasing the cytotoxicity of the alpha-synuclein in the familial mutations of the alpha-synuclein in Parkinson's disease models.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:

Project/Performance Site Congressional Districts:

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Meshal Aldhubayb	POSITION TITLE
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Majmaah University	B.S.	2014	Medical Laboratory Science
Hood College, Frederick, MD	M.S.	2019 (anticipated)	Biomedical Science

A. Positions and Honors

N/A

B. Selected peer-reviewed publications (in chronological order)

N/A

C. Research Support

N/A

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Hood College

Clinical:

N/A

Animal:

N/A

Computer:

Hood College

Office:

Hood College

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

. Centrifuge
. Tissue culture Hood
. Incubator
. Cell countess
. fluorescence microscopy

The following consumables will be purchased with grant funding:

. SH-SY5Y cell line
. Dulbecco's Modification of Eagle's Medium (DMEM)
. Fetal bovine serum FBS
. Penicillin
. Streptomycin
. Puromycin
. Neomycin
. Hygromycin B.
. 24 well plate
. Plasmids
. lipofectamine 2000 reagent

SPECIFIC AIMS

Parkinson's disease is a slow aggressive neurological disorder characterized by a loss of dopamine and formation of aggregates neuronal cytotoxic protein called alpha-synuclein. In order to prevent the alpha-synuclein aggregation Rothaug and colleagues have demonstrated the Lysosomal Integral Membrane Protein type-2(LIMP-2) can reduce the cytotoxicity of the alpha-synuclein. This proposal is to investigate the role of LIMP-2 in decreasing the cytotoxicity of the alpha-synuclein in the familial mutations of the alpha-synuclein in Parkinson's disease cell models.

Aim 1: Determine if plasmids that encode mutated and wild- type alpha-Synuclein with aggregation and non-aggregation phenotype are expressed.

This project will use the cell culture, human SH-SY5Y neuroblastoma cells, to study alpha-synuclein aggregation. The cell line SH-SY5Y will be transfected with plasmids that contain Tet-On in order to control the cytotoxicity of alpha-synuclein aggregation. We will use fluorescence microscopy to examine the protein expression. In order to determine the concentration of the alpha-synuclein we will use Enzyme-Linked Immunosorbent Assay (ELISA) technique.

Aim2: Measure aggregation protein in familial mutations and wild-type of alpha-synuclein.

In this aim, Fluorescence Resonance Energy Transfer (FRET) will be used to define the aggregation of alpha-synuclein with each familial mutation and wild- type. Then the rate of aggregation in each cell line will be quantified relative to alpha-synuclein expression in the same cell line that will be found in aim 1 to determine which of the

familial mutations is more aggressive than others.

Aim 3: Can lysosomal integral membrane protein type-2 (LIMP-2) expression promote clearance of alpha-synuclein aggregation?

In this aim, LIMP-2 will be expressed after the plasmids are transfected in selectable cell lines. We will use ELISA to determine the concentration of LIMP-2 expression in each cell line. The effect of the expressed LIMP-2 plasmid on alpha-synuclein aggregation will be determined by using the FRET method. To answer the question, we will compare the cell lines which are overexpressing LIMP-2 with the cell lines which are not.

BACKGROUND AND SIGNIFICANCE

Parkinson's disease (PD) is a cumulative neurological disorder characterized by loss of dopamine in nerve cells in the substantia nigra and the formation of neuronal protein aggregation such as alpha-synuclein, Lewy Bodies and Lewy Neurites. The result from a decrease in dopamine concentration in basal ganglia is the locomotion defects known as Parkinson's symptoms. In 1817, James Parkinson was the first to describe the clinical features of Parkinson's syndrome that was later afforded his name. At that time, Parkinson's disease was known as paralysis agitans. In late 19th century, Charcot gave privilege to Parkinson by changing the name of paralysis agitans to what we today know as Parkinson's disease. In 1960, Ehringer and Hornykiewicz discovered that Parkinson's patients have markedly decreased the concentrations of dopamine. Recently, contributing factors that lead to death of dopaminergic and non-dopaminergic cells in the brains of patients with Parkinson's disease include genetic mutations, abnormal or unfolded proteins by the ubiquitin- proteasome and the autophagy- lysosomal systems, mitochondrial dysfunction inflammation, and increased oxidative stress (Jankovic,2008). Incidence of Parkinson's disease is 1% of the population over the age of 60, and the incidence increases to 5% of the population over the age of 85.

Incidence of Parkinson's disease in the US from 1995 to 2005 reveal that age plays a significant role in PD. Approximately, 1.6% of the elderly population have been diagnosed with PD per 100,000 Medicare beneficiaries over the age of 65. It shows that when the age increased, the possibility to have PD will increase as well. In addition,

incidence is affected by sex and race among PD patients (Table 1) (Willis *et al*. 2010).

As a result from losing dopamine, Parkinson's patients are characterized by slow movement.

Table 1: Sex and Race different Parkinson's disease incidence and annual incidence per100,000 by ethnic group (Willis *et al* .2010).

Race	1995	2000	2001	2002	2003	2004	2005	Mean ± SD	Prevalence or incidence ratio (95% CI)
White									
Males									
Prevalence	1,963.67	2,253.31	2,231.67	2,203.38	2,187.41	2,159.57	2,178.26	2,168.18 ± 95.64	–
Incidence	–	–	–	537.36	569.71	563.26	571.35	560.42 ± 15.76	–
Females									
Prevalence	1,247.94	1,419.43	1,414.31	1,407.52	1,397.26	1,379.77	1,379.78	1,378.00 ± 59.44	–
Incidence	–	–	–	367.70	387.64	383.20	381.44	379.99 ± 8.60	–
Both sexes									
Prevalence	1,505.46	1,723.43	1,715.86	1,703.34	1,693.74	1,675.62	1,683.99	1,671.63 ± 75.18	Ref.
Incidence	–	–	–	450.57	454.63	450.24	452.03	451.87 ± 2.00	Ref.
Black									
Males									
Prevalence	1,039.55	1,269.05	1,281.34	1,314.08	1,311.14	1,295.80	1,341.12	1,264.58 ± 101.97	–
Incidence	–	–	–	409.31	422.87	428.96	447.53	427.17 ± 15.87	–
Females									
Prevalence	749.45	924.402	919.17	939.22	945.153	949.42	989.99	916.69 ± 77.24	–
Incidence	–	–	–	301.72	319.72	328.16	341.33	322.73 ± 16.59	–
Both sexes									
Prevalence	848.86	1,042.10	1,041.59	1,068.18	1,072.28	1,069.63	1,112.27	1,036.41 ± 86.01	0.58 (0.575–0.581)
Incidence	–	–	–	354.10	354.64	361.93	377.02	361.92 ± 10.68	0.74 (0.732–0.748)
Hispanic									
Males									
Prevalence	1,583.95	1,787.17	1,790.35	1,892.74	1,921.25	1,911.72	1,986.20	1,839.05 ± 133.36	–
Incidence	–	–	–	512.57	569.04	519.14	553.32	538.52 ± 27.08	–
Females									
Prevalence	1,201.13	1,337.8	1,327.65	1,314.08	1,361.15	1,411.30	1,463.02	1,345.16 ± 82.25	–
Incidence	–	–	–	390.78	436.30	439.23	442.90	427.30 ± 24.50	–
Both sexes									
Prevalence	1,356.59	1,513.07	1,511.76	1,562.38	1,581.16	1,608.43	1,671.32	1,543.53 ± 99.33	0.89 (0.881–0.896)
Incidence	–	–	–	462.9	486.92	468.14	486.29	476.06 ± 12.36	1.07 (1.047–1.084)
Asian									
Males									
Prevalence	1,313.19	1,344.10	1,307.56	1,401.90	1,463.11	1,448.29	1,468.02	1,392.31 ± 70.40	–
Incidence	–	–	–	382.79	424.86	392.59	407.00	401.81 ± 18.30	–
Females									
Prevalence	971.64	904.53	922.62	968.39	975.95	984.36	1,019.87	963.91 ± 38.74	–
Incidence		–	–	293.63	279.01	292.27	306.32	292.81 ± 11.16	–
Both sexes									
Prevalence	1,113.23	1,085.96	1,080.69	1,144.22	1,173.45	1,171.76	1,200.61	1,138.56 ± 46.47	0.62 (0.617–0.631)
Incidence	–	–	–	338.34	338.60	332.69	346.94	339.14 ± 5.87	0.69 (0.657–0.723)

After Alzheimer's, Parkinson's disease is the most common neurodegenerative disorder. Age is considered to be the primary risk factor for Parkinson's disease. In addition, ethnicity is a risk factor for Parkinson's disease. In fact, in the USA, highest incidence of population who have Parkinson's disease is Hispanic people of ethnic origin, followed by non-Hispanic Whites, Asians, and Blacks. Environmental factors can also be a risk factor. The environmental factors that lead to increased risk include: pesticide exposure, prior head injury, rural living, β-blocker use, agricultural occupation, and well-water drinking. The environmental factors that lead to a decrease in risks are: smoking, coffee drinking, non-steroidal anti-inflammatory drug, calcium channel blocker, and alcohol consumption (Figure 1) (Kalia *et al.* 2015).

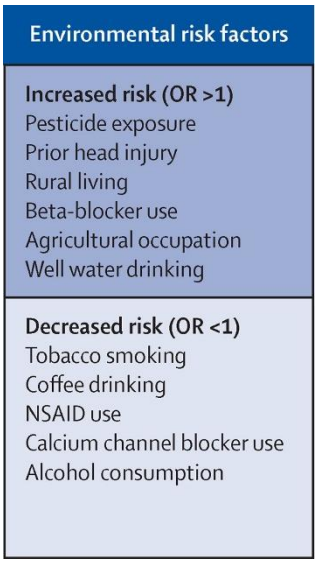


Figure 1: Environment risk factors for development of Parkinson's diseases (*Kalia et al.* 2015).

Parkinson's disease (PD) is a progressive neurological disorder characterized by a huge number of motor and non-motor features that can impact on function to a unstable degree. Clinical feature of PD can be classified into four group TRAP. Tremor at rest, Rigidity, Akinesia (also known as bradykinesia) Postural instability. Bradykinesia, which

means sluggish movement is a significant symptom for clinical feature of Parkinson's disease. Secondly, rest tremor is easily to be recognized and commonly found in PD patients. Thirdly, rigidity locate in neck and shoulder. Finally, contain postural deformities (Jankovic, 2008).

Diagnosis of Parkinson's disease occurs with the start of motor symptoms over time 0 years, however; it can be preceded by a premotor phase of 20 years or even more. This prodromal phase is characterized by specific non-motor symptoms. In early stage of the Parkinson's disease diagnosis clinical feature includes bradykinesia, rigidity, and tremor. In late phase, it may lead to sleep behavior disorder, and psychosis (Kalia *et al*. 2015) (Figure 2).

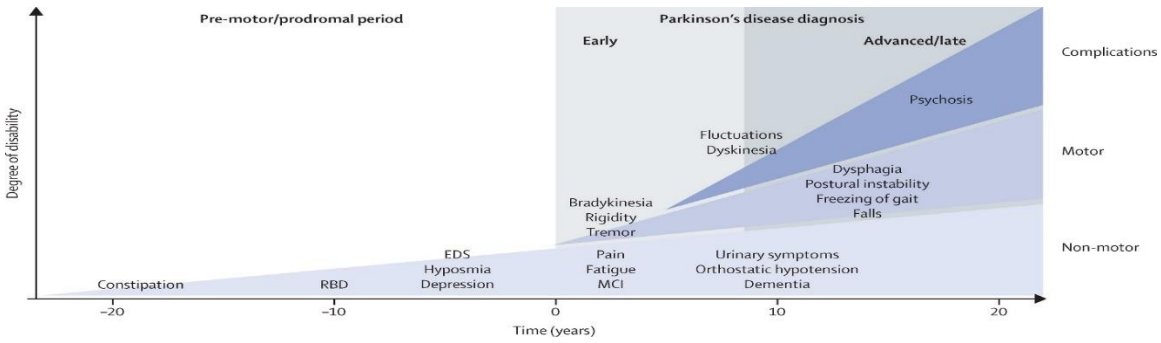


Figure 2: Clinical symptoms and time course of Parkinson's disease progression (Kalia, 2015).

Previous studies reported that Parkinson's disease was thought to be a result of environmental factors; however, recently the researchers found that genetics can play a significant role with environmental factors forming complexity of Parkinson's disease (Kalia *et al*. 2015). Researchers have focused on the concentration of dopamine and the direct stimulation of dopamine receptors as possible ways for treatment in Parkinson's disease, PD does involve neurotransmitters other than dopamine.

The researchers have found that the aggregation of alpha-synuclein is strongly linked genetically and pathologically to Parkinson's disease. Alpha-synuclein belongs to the protein family called synuclein which have beta and gamma (Figure 3). Both alpha and beta are found in nerve terminals in the brain while gamma is found basically in peripheral nerves system (Fink, 2006.). Even though the mechanism of the Parkinson's disease pathogenesis is not clearly understood, the presence of alpha-synuclein aggregation plays a significant role in PD. The function of alpha-synuclein is unknown (Ulrih *et al*, 2008).

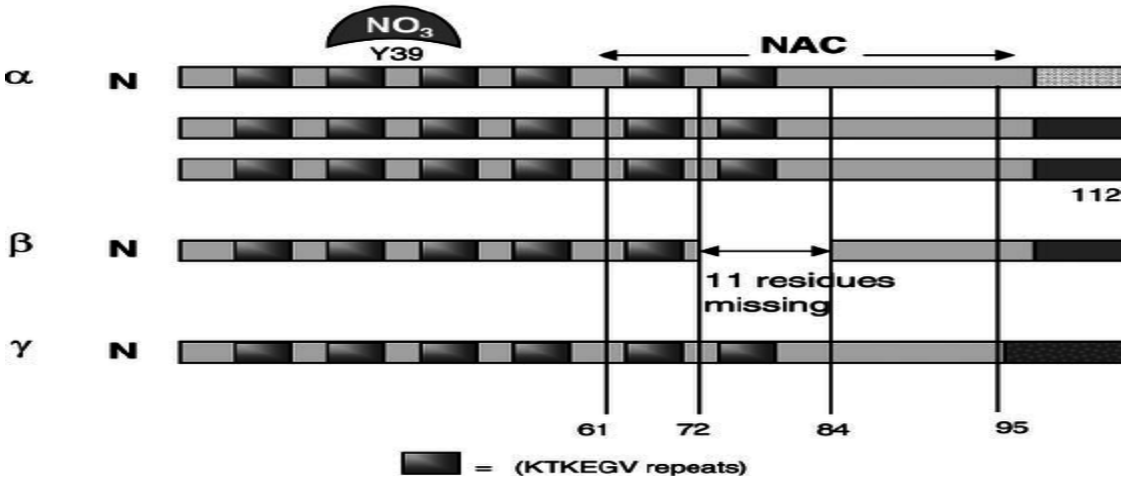


Figure 3**:** Sequence homology among the synuclein protein family. The synucleins all share a conserved N-terminal region, which contains 5– 6 repeats of the KTKEGV consensus sequence (Ulrih *et al*, 2008).

Alpha-synuclein is a 140 amino acid protein that is located in the synaptic vesicles in presynaptic nerve terminal and it has shown to interact with membrane on both in vivo and in vitro (Marques and Outeiro, 2012). Because the presence of a hydrophobic 12 amino-acid sequence in the central part of the protein requires the oligomerization and fibrillization of alpha-Synuclein, deletion or disruption of this domain blocks the capacity

of alpha-synuclein to form amyloid fibrils (Figure 4) (Marques O and Outeiro, 2012).

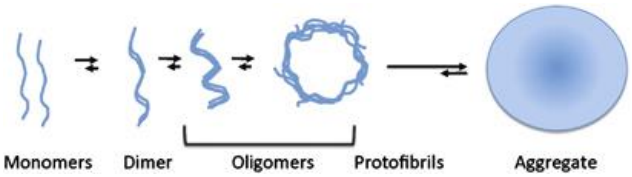


Figure 4: Schematic representation of the alpha-synuclein aggregation process. Monomeric forms of alpha-Syn associate to form dimers and oligomers that grow into protofibrils and, finally, form mature fibrillar structures (Marques and Outeiro, 2012).

Although the physiological function of alpha-synuclein is not fully understood, there is evidence suggesting that alpha-synuclein has a role in neurotransmitter release. Mice lacking the synaptic co-chaperone cysteine-string protein alpha (CSPa) present progressive neurodegeneration and impairment in synaptic function. Secretion of alpha-synuclein is not fully understood; however, there is evidence that shows that alpha-synuclein is secreted by non-classical ER/Golgi-independent protein export pathway (Figure 5). Alpha- synuclein can be secreted both in it is monomeric or aggregated forms by non-classical exocytic or endocytic pathways. In the first way, alpha-synuclein can be directly integrated into secretory vesicles and subsequently released by exocytosis. In the second way, alpha-synuclein can be translocated to early endosomes. From early endosomes, alpha-synuclein protein can either be released to the extracellular space through the recycling endosome or incorporated to intraluminal vesicles of multivesicular bodies (MBV). MVB cargo including alpha- synuclein can be directed to degradation by fusion with lysosomes or to secretion by fusion with the plasma membrane and release of exosome vesicles (Figure 5) (Marques and Outeiro, 2012).

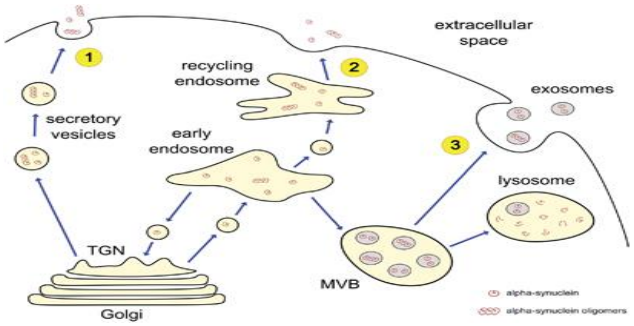


Figure 5: Proposed mechanisms of alpha- synuclein secretion (Marques and Outeiro, 2012).

Extracellular alpha-synuclein plays a critical role in neurotoxic and neuroinflammation. Alpha-synuclein emitted by a dying cell into extracellular space Extracellular alpha-synuclein stimulates nearby astrocytes and microglia, eliciting glial pro-inflammatory activity. Activation microglia produce pro-inflammatory elements: cytokines, reactive oxygen species, and, nitric oxide which is cytotoxic to neurons. Alpha-synuclein can be transferred between neurons, resulting alpha-synuclein aggregation process to spread and decrease the viability of the recipient neuron (Figure 6) (Marques and Outeiro, 2012). Previous study confirm that genetics has a significant role in formation of alpha-synuclein aggregation.

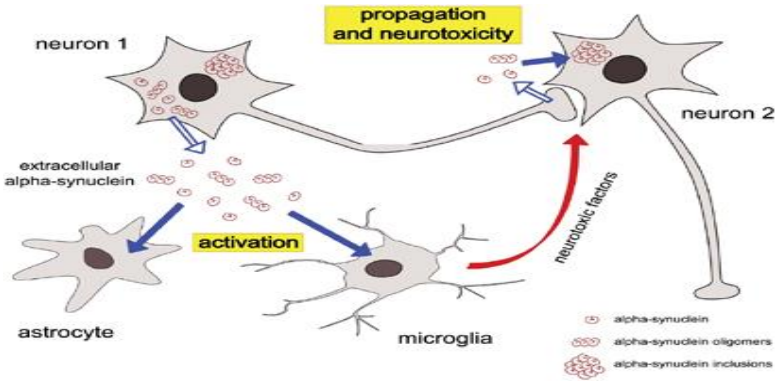


Figure 6: The role of extracellular of alpha-synuclein (Marques O and Outeiro, 2012).

Familial Parkinson's disease is an autosomal dominant disorder caused by missense mutations and multiplications of the SNCA gene, encoding α-synuclein. Studies of families with history of Parkinson's disease have included the identification of familial mutations (A30P, E46K, H50Q, G51D, A53T). Alpha-synuclein is a 140-amino-acid protein containing three domains: The N-terminal amphipathic domain, the non-amyloidogenic, which includes a hydrophobic core, and the C-terminal acidic domain. Parkinson's disease-related mutations (A30P, E46K, H50Q, G51D, A53T and A53E) are located at the amphipathic domain (Figure 7) (Devoto and Falzone , 2017). Preventing aggregation of alpha-synuclein is considered to be one possible way to increase the dopamine concentration in Parkinson's disease.

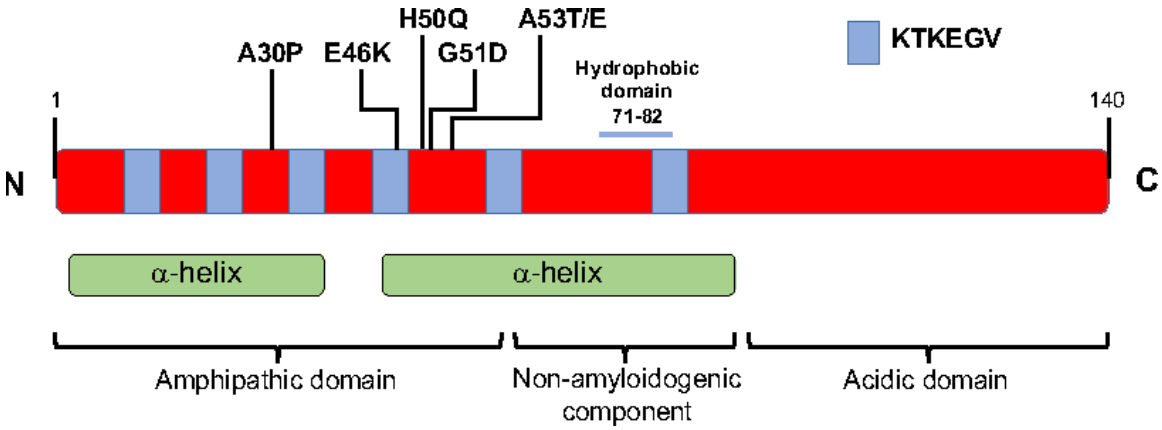


Figure 7: Alpha-synuclein structure (Devoto and Falzone , 2017).

Previous researchers have tried to prevent alpha-synuclein aggregation. Recently, there is evidence LIMP-2 can affect the aggregation of alpha-synuclein by reducing the amount of alpha-synuclein (Rothaug *et al*.2014). The LIMP-2 is the receptor for lysosomal transport of acid hydrolase β-glucocerebrosidase (GC). The gene encode LIMP-2 is *SCARP2*. In LIMP-2–deficient mice increased levels of endogenous

α-synuclein lead to severe neurological deficits and premature death, reducing lysosomal GC activity. Lipid storage distribution, autophagic lysosomal function, and α-synuclein accumulation lead to neurotoxicity of dopaminergic neurons as well as apoptotic cell death and inflammation. In addition, overexpression of LIMP-2 enhances alpha-synuclein clearance and increases lysosomal activity of GC (Rothaug *et al*.2014). To investigate the role of LIMP-2 in the familial mutations of Parkinson's disease, we propose to use cellular models for Parkinson's disease.

Cellular model for Parkinson's diseases is necessary to study therapeutic strategies, however; there is still challenge for cellular model. The challenges for cellular models is the limited information on what causes sporadic Parkinson's disease and therefore, cannot reproduce the entire pathogenesis in a model. Second, because Parkinson's disease pathology is slow or late, and the cell model has to, unlike Parkinson's disease itself, develop pathology quickly and reliably. The neuroblastoma cell line SH-SY5Y is widely considering to be the most common cell line to study Parkinson's disease. The advantages of using SH-SY5Y cell line are: the release of catecholamines, development of neuron-like properties, ease of maintain. Even although differentiation can be difficult, SH-SY5Y is an excellent model for studies with genetic and pharmacological interventions (Falkenburger *et al.*2016). Because SH-SY5Y cell line itself cannot produce aggregation of alpha-synuclein, it is necessary to transfect plasmids that can express the alpha-synuclein and monitor the expression and aggregation through advanced methods.

In this proposal, the main focus is to evaluate if the LIMP-2 overexpression affects the quantity of alpha-synuclein aggregation in familial mutations using Parkinson's disease model.

PRELIMINARY REPORT / PROGRESS REPORT

Previous work defines Parkinson's disease as a cumulative neurological disorder characterized by damage of dopaminergic neurons of the substantia nigra and formation of neuronal protein called alpha-synuclein aggregates known as Lewy bodies. Previous work show a decrease in dopamine concentration in the basal ganglia that drives the locomotion defect which is known as Parkinson's symptoms (Jankovic, 2008). Parkinson's disease was thought to be a result of environmental factors; however, recently the researchers found that genetics can play a significant role with environmental factors impacting the complexity of Parkinson's disease. Researchers have found that the aggregation of alpha-synuclein is strongly linked genetically and pathologically to Parkinson's disease (Zhou, 2006). Parkinson's disease is an autosomal dominant disorder caused by missense mutations and multiplications of the SNCA gene, which encodes α-synuclein. Studies of families with a history of Parkinson's disease have included in the identification of familial mutations (A30P, E46K, H50Q, G51D, A53T) (Devoto, 2017). Previous researchers have tried to prevent alpha-synuclein aggregation. Rothaug and his colleagues demonstrate that in LIMP-2 deficient mice, increased levels of endogenous α-synuclein lead to severe neurological deficits and premature death, as a result from reduced lysosomal GC activity. This resulted in lipid storage distribution and autophagic lysosomal function and α-synuclein accumulation leading to neurotoxicity of dopaminergic neurons as well as apoptotic cell death and inflammation. In addition,

overexpression of LIMP-2 enhances alpha-synuclein clearance and increases lysosomal activity of GC. Prevention of aggregation of alpha-synuclein by overexpression of LIMP-2 may be a way to increase dopamine concentration and contribute to treating (Rothaug,2014).

RESEARCH DESIGN / METHODS

Alpha-synuclein (AS) aggregation is widely considered to play a critical role in the progression of Parkinson's disease. The hypothesis of this project is to estimate if LIMP-2 can prevent or slow alpha-synuclein aggregation in cases of familial mutations of the alpha-synuclein gene. Briefly, the first aim demonstrates of the overexpression (familial mutation and wild-type alpha-synuclein overexpressed in SH-SY5Y cell line). The second aim will evaluate kinetics of aggregation of the alpha-synuclein by FRET. In the third aim, overexpression of LIMP-2 will be evaluated on the prevent of alpha-synuclein aggregation. We will design the plasmids that encode LIMP-2 expression and transfect them into cell lines. The final expected result of the research is that expressed LIMP-2 plasmid in cell lines will show lower concentration of the alpha-synuclein compared with cell line which does not express the LIMP-2.

Aim 1: Determine if plasmids that encode mutated and wild-type alpha-synuclein with aggregation and non-aggregation phenotypes are expressed.

Cell culture:

Cell culture provides model systems for studying the normal physiology

and biochemistry of cells like metabolic studies, aging, the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. This project will use the cell culture model human SH-SY5Y neuroblastoma cells (Manassas, VA). The cells will be maintained in Dulbecco's Modification of Eagle's Medium (DMEM) culture medium (Invitrogen) (Manassas, VA), supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37 C with 5% CO2. SH-SY5Y is selected for this project because it is of human origin, catecholaminergic, neuronal properties, and ease of maintenance (Figure 8).

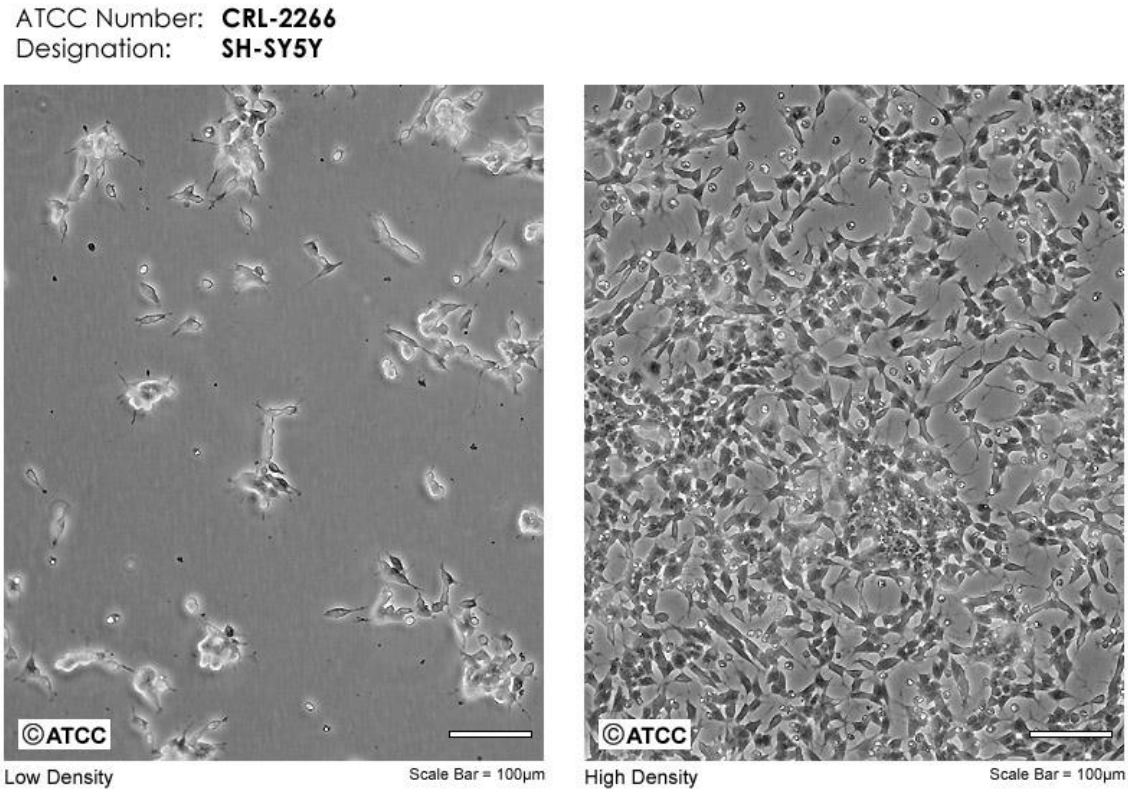


Figure 8: The morphology of the SH-SY5Y neuroblastoma cells under microscopy (ATCC)

Plasmids:

Studies of families with a history of Parkinson's disease have been identified of familial mutations (A30P, E46K, H50Q, G51D, A53T) (Devoto and Falzone, 2017). Plasmids will be purchased from Origene (Catalog # 30130) (Rockville, MA). By using custom request form for clone modification in www.origene.com website, the plasmids can be designed to have the gene encoding alpha-synuclein with different familial mutations (A30P, E46K, A53T, G51D, H50Q) and wild-type as a control. Other elements in plasmid will include, Cyan Fluorescent Protein CFP, Yellow Fluorescent Protein YFP, and selectable marker such as puromycin and neomycin. In addition, the plasmids also included Tet On, TA, and promoters. The end of this step will be total with 12 plasmids summarize in Table 2. Plasmids will be transfected into cell lines in the next step.

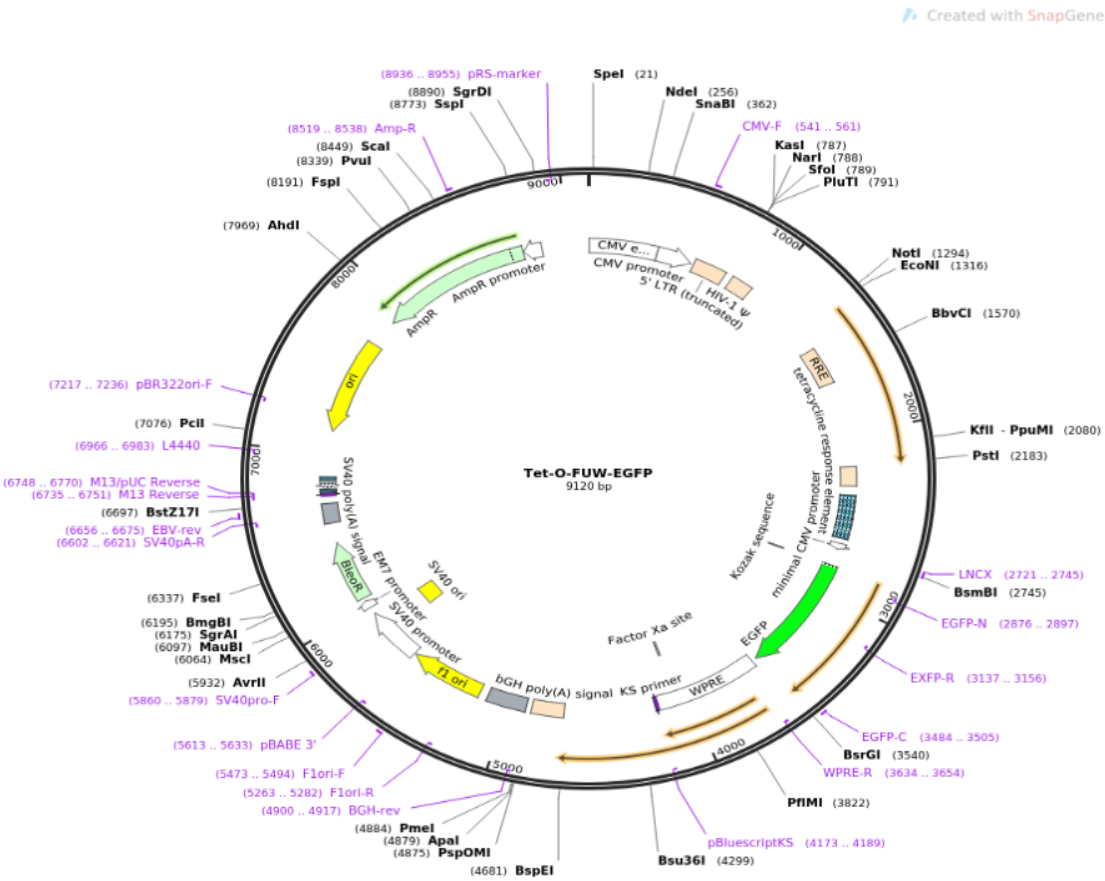
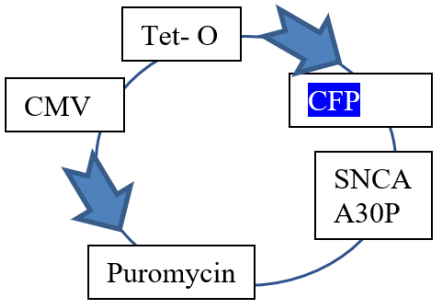
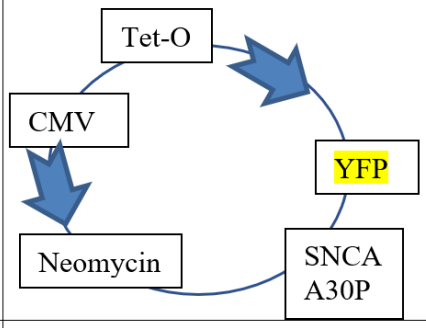
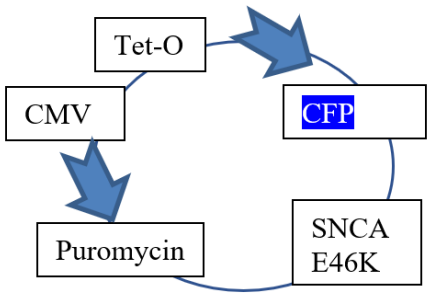
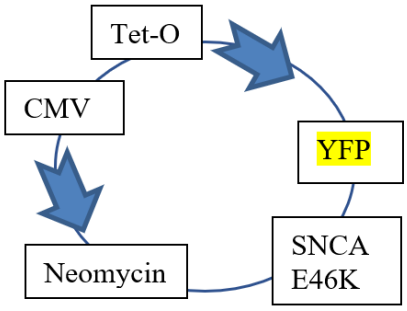
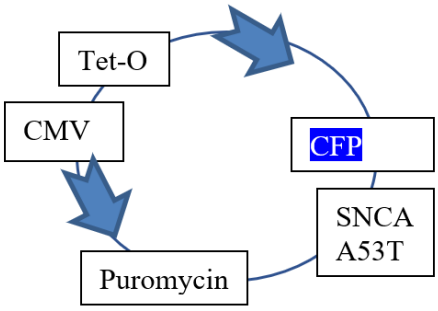
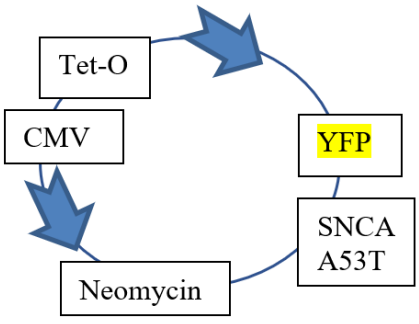
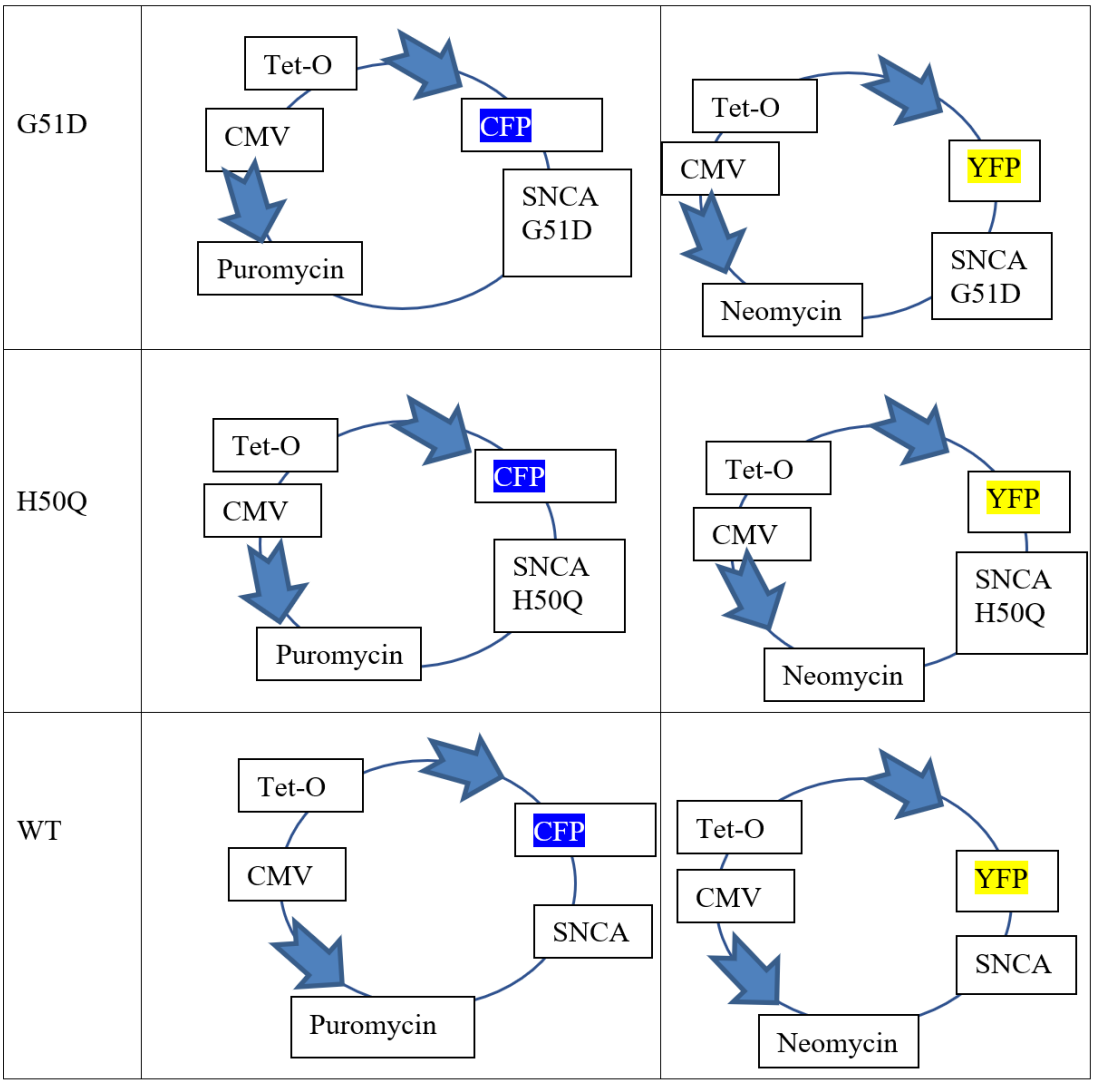


Figure 9: Structure example of plasmid (not the actually our plasmids) (addgene).

Table 2: The elements of plasmids in each cell line

Name Of the Cell lines:	Elements of plasmid which contain the CFP	Elements of plasmids which contain the YFP
A30P		
E46K		
A53T		



Determine the optimal selection antibiotic concentration:

The first important step for stable cell line generation is to determine the favorable antibiotic concentration for selecting stable cell colonies. Kill curve is a dose-response experiment where the samples of challenged increasing doses cells are undergoing to rising amounts of antibiotic to determine the minimum antibiotic concentration necessary to kill all the cells over a period of time usually (3-5 days). Kill curve result depends on type of antibiotic and cell line. The antibiotics that will be evaluated for this experiment are puromycin, neomycin, and hygromycin B. Cells

expressing gene for antibiotic resistance from the plasmids will survive. By using 24well plate, the cells can be examined to determine the minimum concentration of antibiotic that require to killing the cells in two and three days (Figure 10).

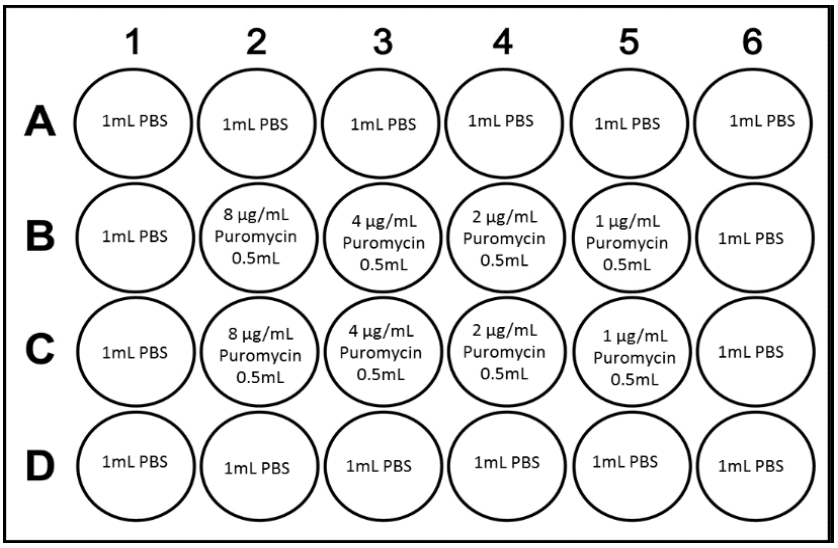


Figure 10: Plate puromycin will be used in different concentration in different cell volume to measure the minimum concentration of antibiotic that can kill the cell by examining the cells under microscope, usually take 3-5 days.

Transfection:

Factors that influence the efficiency of transfection are cell type, cell health and viability, confluency, media, and type of transfection. Cells are transfected with plasmid DNA (Figure 11) by using lipofectamine 2000 reagent, which contains liposome forming cationic lipids that complex with negatively charged nucleic acid molecule to give them opportunity to cross cell membrane. The main purpose of using this method is to increase the efficiency of plasmid DNA uptake by cells. After 48hr, antibiotics

are added to select for surviving cells. Cells that survive are expected to express the antibiotic gene (Cat. No.3585) (Corning, NY).

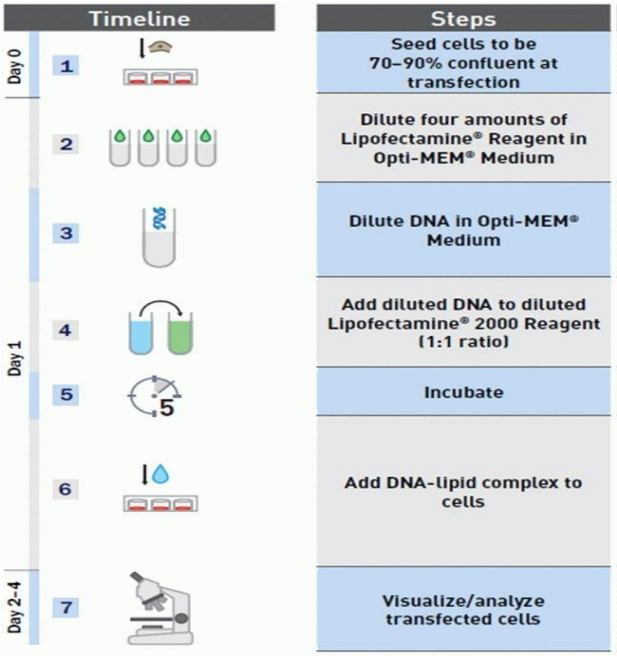


Figure 11: The process of lipofectamine 2000 (Thermo Fisher).

Tet-On Systems for Doxycycline-inducible plasmids Expression:

The Tet-On system is a regulatory system that allows activation of gene expression by the addition of doxycycline. Main purpose of using this method is to increase the amount of the alpha-synuclein in a short time and control cytotoxicity of alpha-synuclein. The Tet-On system depends on a reverse tetracycline-controlled trans activator *rtTA*. *rtTA* is a fusion protein which contains the TetR repressor and the VP16 transactivation domain; however, four amino acids change in the tetR DNA binding moiety alters *rtTA's* binding characteristic allowing it to recognize the tetO sequences in the target transgene in the existence of the doxycycline effector. As a result, the Tet-On system regulates target gene (alpha-synuclein) stimulated by *rtTA* only in the

presence of doxycycline. The plasmids in this project, contain the TetO and rtTA in the backbone on the plasmids. So, when adding the doxycycline, the gene will be expressed to determine the optimal concentration for doxycycline when the gene turns on can be detectable through fluorescence microscopy by monitoring the yellow and cyan colors.

Single cell cloning:

This method is widely used in cell culture. The main purpose of using this technique in this project is to control the plasmids transfected into the cell lines by using 96 well. Plates Single cell cloning gives a uniform population, and is a critical step, because there is no way to control for insertion site number of the plasmids in cells. In each cell line, we will be sure that the ratio of plasmid YFP and to CFP is 1:1. The cell cloning kit will be purchased through Corning, NY.

Enzyme Linked-Immuno-Sorbent Assay ELISA:

Enzyme Linked-Immuno-Sorbent Assay (ELISA) is used to determine the quantity of protein. ELISA is fast, consistent, and easy to analyze. We will use the ELISA to determine the concentration of alpha-synuclein in each of the familial mutation and wild-type. The alpha-synuclein human ELISA kit (Catalog # KHB0061) will purchase through Thermo Fisher. The type of ELISA that will use in this project is a sandwich ELISA (Figure 11).

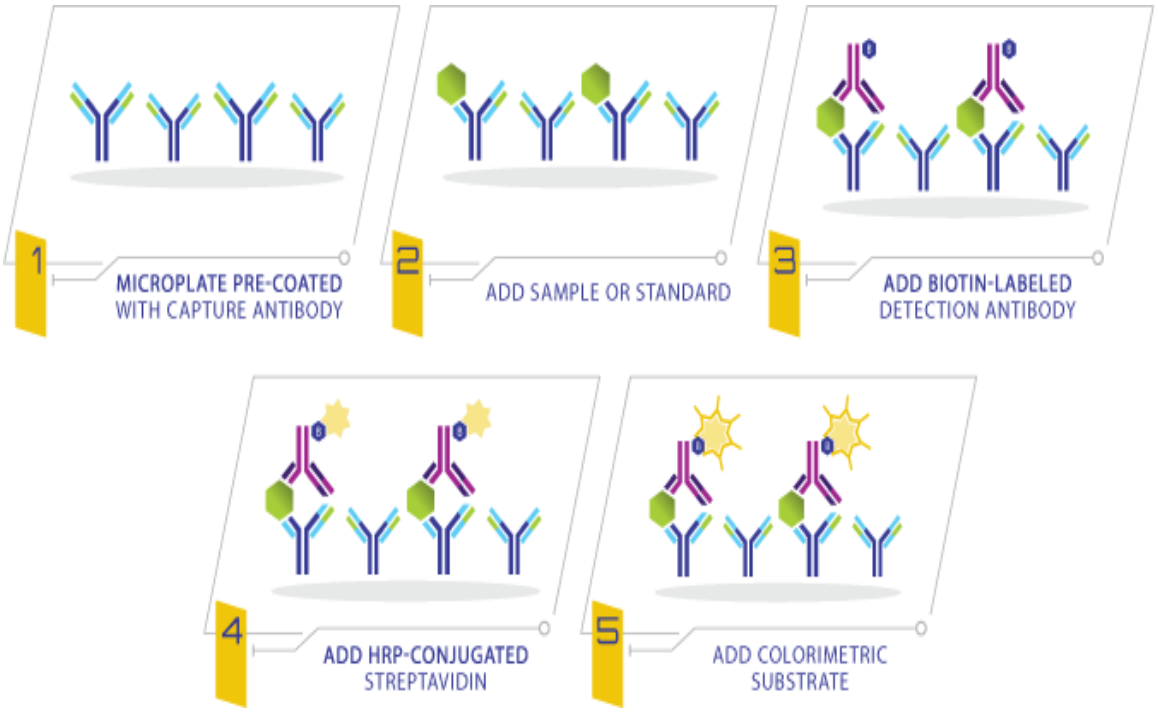


Figure 12**:** The principle of the sandwich ELISA (Thermo Fisher).

Expected result:

The cell lines transfected with plasmids express selectable markers. To confirm gene expression from the plasmids, we will use fluorescence microscopy to detect fluorescence markers. I would expect also, the total concentration of alpha-synuclein in cell lines based on ELISA results.

Aim2: Measure aggregation protein in familial mutations and wild-type of alpha-synuclein

Fluorescence Resonance Energy Transfer (FRET):

The development of fluorescence imaging technologies especially green fluorescent protein gives us opportunity to visualize molecular signals at subcellular levels in live cells. Earlier assays to detect and measure molecular signals usually required to killing cells, which impacts the information of the cells. With fluorescent protein and biosensor-based fluorescent protein (FPs), molecular signals at different subcellular location can be observed in live cells without damaging the cells. FRET is a quantum mechanics phenomenon where two fluorescent proteins, donor and acceptor, with the donor emission spectrum overlapping the excitation. The energy can transport when the donor and the acceptor are close to each other with proper close angles. Fluorescent proteins have been derivatized based on Enhanced Green Fluorescent Protein EGFP to include additional different colors including blue (BFP), cyan (CFP) and yellow (YFP). In this experiment, we chose cyan (CFP) and yellow (YFP) to measure the wavelength of aggregation in the plasmids and examine the result through biosensor for each familial mutation and wild-type and measure the time (Figure 12) (Lui et al.2010).

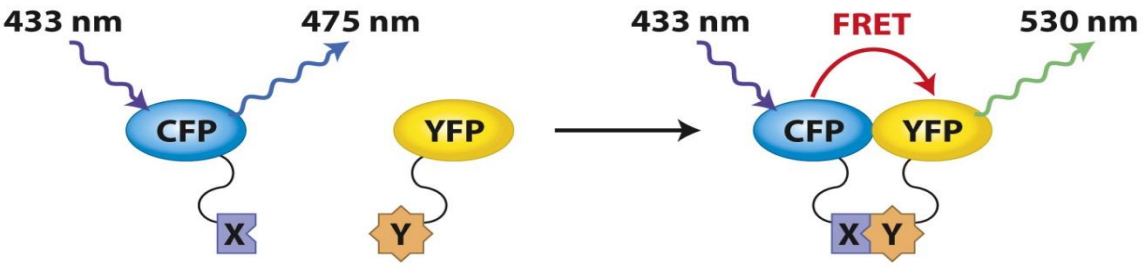


Figure 4-24a
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Figure 13: Simple principle of FRET, the X, and Y in this figure means the same cell

Expected result:

The rate of aggregation will be different for each of the familial mutation relative to the wild type. The A53T mutation should promotes rapid aggregation. Based on previous published results. In this project, the rate of aggregation for each mutation divided by total concentration of alpha-synuclein for each familial mutation will provide quantitation.

Aim 3: Can Lysosomal integral membrane protein type-2 (LIMP-2) expression promote clearance of alpha-synuclein aggregation?

Lysosomal integral membrane protein type-2 is a receptor for lysosomal transport of the acid hydrolase β-glucocerebrosidase (GC). Expression of the LIMP-2 leads to increase the activity of β-glucocerebrosidase (GC) and as a result, the activity of alpha-synuclein will decrease. In this aim, we are going to investigate the effect of LIMP-2 on each of the familial mutation compared with wild-type.

Cell culture:

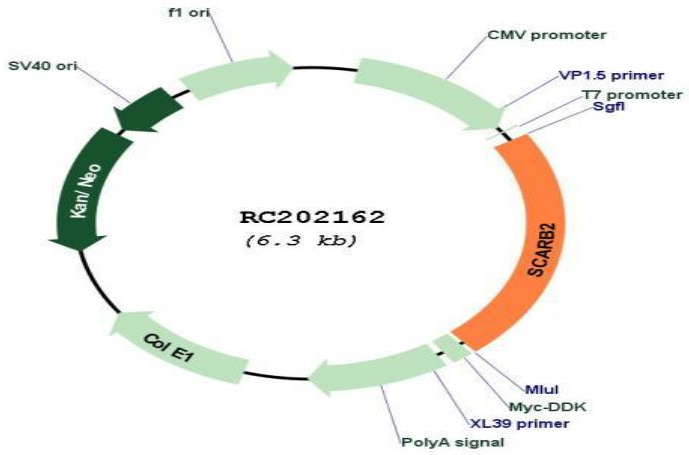
Six cell lines developed in the first aim will be maintained in Dulbecco's Modification of Eagle's Medium (DMEM) culture medium (Invitrogen), supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37 C with 5% CO2.

Plasmids:

The plasmids to be transfected will contain LIMP-2. Plasmids can be designed to have the gene encoding LIMP-2 which is SCARB2 and counting the select marker for LIMP-2 which is hygromycin B. The plasmids also have Tet-On system which will increase plasmids expression. Cell lines without LIMP-2 plasmid transfected

will be considered the negative control. The plasmids for LIMP-2 will be purchased through Origene LIMPII(SCARB2) (NM_005506) Human Tagged ORF CloneCAT#: RC202162 (Rockville, MD) (Figure14).

Figure13: Map of the LIMP-2 plasmid (not actual our plasmid) (Origene).



Enzyme Linked-Immuno-Sorbent Assay ELISA:

The main goal of using this technique is to determine the concentration of LIMP-2 in six cell lines which are approved in first aim. The ELISA kit will be purchased through Thermo Fisher (Catalog # 702770).

Fluorescence Resonance Energy Transfer (FRET):

In this experiment, we will have two main groups of cell lines, one with plasmid expression of LIMP-2 and a second group without plasmid expression of LIMP-2 will be considered as a control. FRET biosensor detection will show how LIMP-2 impacts

alpha-synuclein aggregation relative to each familial mutation. Rapid aggregation form mutation A53T will have the biggest difference between the LIMP-2 and the negative control.

Expected result:

The result from cell lines which express LIMP-2 will decrease the FRET emission while the cell line that does not express the LIMP-2 will show a FRET emission increase. However, the decrease of emission may different among the different familial mutation. Mutation A53P may have the most effect from LIMP-2 plasmid expressed compared with the mutations and wild-type. Determining the role and effectiveness of the LIMP-2could be one of the promising strategies to reduce the amount of alpha-synuclein aggregation, and as a result be a potential method for treating Parkinson's disease.

Aims	Time	Activity
Aim 1	1.0 month	Obtaining the selectable cell lines.
	1.0 month	Obtaining the selectable plasmids.
	1.0 month	Cloning the plasmids.
	0.25 month	Insert the plasmids.
	0.25 month	Culturing and maintaining the cell lines.
	0.25 month	Determine the optimal selection antibiotic concentration
Aim 2	0.25 month	Monitor the plasmids expression.
	0.25 month	Cell viability.
Aim 3	1.0 month	Obtaining the selectable plasmids.
	0.25 month	Insert the plasmids.
	0.25 month	Monitor the plasmids expression

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