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Deciphering Underlying Mechanisms Of Photoreceptor Outer Segment Degradation; Involvement Of Intracellular Trafficking

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Deciphering Underlying Mechanisms of Photoreceptor Outer segment Degradation; Involvement of Intracellular Trafficking

by

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Dedication

To Caleb, my husband, for your support and sacrifice through these years as I have pursued my passion. Thank you for believing in me and being the best dad to our sons while I did science. To my sons, Israel, Josiah & Isaiah- thank you for making me a mommy and for your understanding each time I came home too late to tuck you in at night. To my parents, siblings and friends – thank you for being my voices of encouragement, for praying with me and for me. To my mentor Dr. Chen, thank you for teaching me, believing in me, for recognizing me and pushing me to be the best I can be. Most importantly, all I am and ever hope to be in life I owe it to my Lord and Savior Jesus Christ. The constant hope and source of all joy in my life.

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Deciphering Underlying Mechanisms Of Photoreceptor Outer segment Degradation; Involvement Of Intracellular Trafficking

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Age related macular degeneration (AMD) is the leading cause of vision loss among adults 50 and older in the United States. AMD is characterized by the degeneration of photoreceptors and retinal pigment epithelium (RPE) cells in the macula region of the retina. RPE maintain the health of the retina by providing support to the photoreceptor cells and the retina through various tasks. One major task RPE cells are involved in, is daily uptake, trafficking and degradation of shed photoreceptor outer segment (POS). It has been shown that with age and as a result of cellular stress this process can become impaired. Impairment in this unique phagocytic process can lead to degeneration of the RPE cells as well as the adjacent photoreceptor cells. In order to understand the pathogenesis of AMD and other retinal degenerative diseases it is important to uncover the molecular factors that are involved in the phagocytic activity of RPE uptake and clearance of POS. Lack of molecular methods and assays have limited our knowledge of these factors. In this project I developed a novel method which is used to uncover new factors specifically involved in POS trafficking in the RPE. Foster Resonance Energy Transfer or FRET is an energy transfer mechanism used to monitor interactions between proteins. This method was utilized to monitor movement of phagocytosed POS within the RPE to the lysosome. Using

FITC labeling of POS and Lysotracker labelling of lysosomes I developed a novel assay to monitor the energy transfer of FITC labelled POS to Lysotracker labelled lysosome. The FRET assay is validated using Chloroquine treatment of ARPE-19 cells and knockdown of Atg5. This unique assay gives specific information regarding the trafficking of POS to the lysosome within the RPE. The assay is useful for uncovering novel factors involved in the trafficking stage of POS phagocytosis. With the developed assay I show the role of vesicular protein sorting subunits 18 and 11 in POS trafficking. In VPS18 and VPS11 siRNA knockdown ARPE-19 cells, POS trafficking is impaired. The working model suggests that disruption of endocytic pathway complex, HOPS and COVERT leads to impaired trafficking in RPE cells. The developed trafficking assay uncovered the role of the endocytic pathway in POS phagocytosis in RPE.

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ABCA4	ATP- binding cassette transporters -4
A2E	N-retinylidene-N-retinylethanolamine
AP2	Adaptor Protein 2
atRAL	all-trans-retinal
AMD	Age – related macular degeneration
BAR	Bin/Amphiphysin/Rvs domain containing proteins
CavME	Caveolae-Mediated Endocytosis
CCP	Clathrin – Coated Pit
CCV	Clathrin – Coated Vesicle
CFH	Complement Factor H
CFB	Complement Factor B
CIE	Clathrin – Independent Endocytosis
CLIC	Clathrin -Independent Carriers
CME	Clathrin -Mediated Endocytosis
COVERT	class C core vacuole/endosome tethering
CORVET	class C core vacuole/endosome tethering
EE	Early Endosome

ESCRT	endosomal sorting complex required for transport
FRET	Foster Resonance Energy Transfer
FITC	Fluorescein Isothiocyanate
FAK	Focal Adhesion Kinase
GEEC	GPI-AP enriched endocytic compartments
IFN- γ	Interferon Gamma
HOPS	Homotypic fusion and protein sorting
LRAT	Lecithin Retinol Acyltransferase
MerTK	Mer Tyrosine Kinase
MFG – E8	Milk Fat Globule EGF – factor E8
MVB	Multivesicular body
NBD	7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl0-1,2-Dihexadecanoylsn-Glycero-3 Phosphoethanolamine, Triethylasmmonium Salt.
NLRP3	NLR family pyrin domain containing 3
PEDF	Pigment Epithelial Derived Factor
PIP	Phosphatidylinositol phospholipid
POS	Photoreceptor Outer Segment
PPR	Pattern Recognition Receptor
RCS	Royal College of Surgeons rats

RDH8/12	Retinol Dehydrogenase 8/12
RDH5	Retinol Dehydrogenase 5
RPE	Retinal Pigment Epithelium
RPE 65	Retinoid isomerohydrolase RPE65
SNARE	Soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor
SOD	Superoxide Dimutase
TGN	Trans- Golgi network
TNF-α	Tumor Necrosis Factor Alpha
TLR	Toll- like Receptor
VEGF	Vascular Endothelial Growth Factor
VPS	vacuolar protein sorting
VPS18	Vacuolar protein sorting subunit 18
VPS11	Vacuolar protein sorting subunit 11

Chapter 1 Development of a quantitative fluorescence assay for measuring efficiency of trafficking of photoreceptor outer segment following phagocytosis by RPE

Introduction

Age related macular degeneration; AMD is a degenerative eye disease which results in a drastic decline of quality of life for those individuals who suffer from it. AMD leads to irreversible vision loss and there is still no cure for this age associated disease. For years researchers have labored to uncover the molecular mechanism of this disease. Although our understanding of the disease is improving there remains significant gaps in knowledge. AMD is believed to be caused by a combination of several genetic and environmental factors, some of which include aging, oxidative damage, impaired photoreceptor outer segment phagocytosis, and complementation dysregulation and inflammation.

A deeper understanding of the molecular machinery involved in the disease could lead us to therapeutic targets for treatment of this debilitating disease. Retinal Pigment Epithelium or RPE, a specialized group of cells within the neurosensory retina are a primary site of injury in AMD. These cells provide numerous support functions to the retina. These post mitotic cells undergo daily phagocytosis of shed photo receptor outer segment (POS). This phagocytic activity is an important aspect in maintenance of retinal homeostasis. In other words, health of the retina is predicated on RPE phagocytic function. Increased oxidative stress due to the daily phagocytosis of POS as well as other age associated changes contribute to dysfunction of the RPE. Inflammation as well as disrupted autophagy and POS clearance all lead to dysfunction of RPE. Deciphering the mechanisms by which RPE properly phagocytize shed POS is important for the discovery of the key

regulators in the process. Limited methods of analyses of the phagocytic process has impaired our ability to uncover new molecules involved in the process. This project focuses on the development of an assay used to elucidate novel regulatory molecules which could be involved in RPE phagocytosis. A novel method to study this process could aid researchers in studying an overlooked area of RPE biology. Discovery of these regulatory molecules could contribute to our understanding of this unique phagocytic process and offer us the framework for novel therapies for retinal degenerative diseases like AMD.

Background

STRUCTURE OF THE EYE

The human eye contains many intricate structures which protect it and ensure visual health. A few pictured in Figure 1.1 are as follows:

1. **Cornea:** the clear dome like area which covers the iris
2. **Lens:** behind the iris and pupil helps with focus
3. **Retina:** Innermost layer of tissue
4. **Macula:** Part of the retina; specialized region responsible for sharp vison and focus
5. **Choroid:** network of vessels that provide nutrients and oxygen to the retina
6. **Sclera:** the white area of the eye; membrane of tendon that protects inner parts of the eye
7. **Optic Nerve:** connects the eye to the brain by sending signals from the eye to the brain to decode the signals into images
8. **Photoreceptor –** Rods and Cones light sensing receptors
9. **Retinal Pigment Epithelium:** Specialized layer of polar cells which function in maintaining retinal homeostasis

The macula is a small area within the retina, measured 5.5 millimeters in diameter, responsible for sharp vision and central vision. This region is most susceptible to degenerative diseases such as AMD (2) (Figure 1.2). The photo sensory part of the eye is composed of retinal photoreceptors, rods and cones as well as a monolayer of pigmented cells, retinal pigment epithelium or RPE (Figure 1.2). The RPE cells play a major role in mainainting retinal homestatis and functionality.

AGE RELATED MACULAR DEGENERATION AND RETINAL DYSFUCTION

Age related macular degeneration (AMD) is a degenerative eye condition caused by deterioration of specialized cells within the macula. It is the leading cause of vision loss and legal blindness in adults 50 and older in the USA (2). As life expectancy continues to increase in the USA and other developed nations, the prevalence of age-related disease continues to rise. As of 2010, AMD affected about 17% of U.S population aged 60-74 and approximately 75% of people 74 or older (NEI). As the population continues to age, it is estimated that by 2020, 3 million people in the US will be affected by AMD (Figure 1.1). This major health problem costs the United States $30 billion annually in loss to the economy besides health-related costs (5). Loss of central vision, associated with AMD can interfere with simple daily tasks such as the ability to see faces, drive, write, read, and cook. AMD is a multifactorial disease which is influenced by environmental and genetic factors (2). Although age is the major risk factor for AMD smoking is believed to double the risk (2, 5). It has also been shown that more Caucasians than African–American or Hispanic Latinos are affected with AMD (2, 5).

AMD can be characterized into different stages depending on disease progression. Early AMD is characterized by the formation of small extracellular deposits of fatty proteins called drusen (Figure 1.3). Drusen, the defining clinical feature of AMD, can be found in between the RPE and Bruch's membranes, as shown by black arrows in the middle panels in Figure 1.3 A & B. Drusen is composed of lipids, protein derivatives and other cellular waste products (49). Drusen deposits build up when the cells ability to clear debris is hampered. Drusen deposits are highly concentrated with lipid components, esterified cholesterol and phosphatidylcholine (49). It has also been reported that other components include apolipoprotein E, amyloid Beta, vitronectin and complement proteins (49). This indicates that the formation of drusen within the retina triggers inflammatory responses. Individuals with early AMD usually have no vision loss. Intermediate AMD is

characterized by the presence of large drusen, pigment changes in the retina and extensive damage to RPE cell layer as seen in Figure 1.3 B. Although most people with intermediate AMD do not have major vison loss, severe visual distortion can happen at this stage. Late AMD is associated with presence of drusen, vison loss and atrophy of RPE cell layer as observed in Figure 1.3 C & D. There ae two types of late AMD: geographic (dry) and neovascular (wet) as referenced in Figure 1.3 C & D. In dry AMD, there is progressive loss of light sensitive cells in the macula and loss of the pigmented epithelium cell layer. This loss of photoreceptor cells and the pigment epithelium cell layer ultimately leads to vision loss. In wet AMD, abnormal blood vessels begin to grow underneath the retina. The blood vessels leak fluid and blood, which can lead to swelling and damage of the retina. In the wet form of AMD, the epithelium cell layer is also damaged extensively. There are no treatments for early AMD and no therapy to restore vision in late AMD. Although AMD is a multifactorial disease deterioration of the specialized RPE plays a key role in the pathogenesis of AMD (1,2).

AMD is a multifactorial disease and as described previously, there are many factors which contribute to the disease eitology. The mechanism by which these risk factors lead to disease can vary molecularly. Visual phototransduction is the process by which visual information is converted to electrical signals via the photoreceptor cells within the retina. The visual cycle is an important process for the conversion of light infromation, however by products from the process can become toxic within the retina. During the visual cycle , light absorbed by photorecptor cells caues the isomerization of 11 – cis retinal to atRAL. atRAl is reduced and trsported as all – trans- retinold by the ATP -binding transported (ABCA4) and all -trans retinal dehydrogenaseses (RDH8/12). atRAl moves into the specliazed RPE cells and is converted to all – trans- retinyl esters by lecithin retionla acyltransferase (LRAT). RPE proteins (RPE 65) isomerizes the esters to 11 – cis retinols which is oxidized by RDH5 to 11 – cis retinal. atRAL is the major cellular waste product of the visiual cycle. As a result of celluar stress and oxidative damage the cell must employ

a well orchestrated waste system to rid itself of reactive oxygen species (ROS) which pose tremendous threat. By-products of the visual cycle contibute to cellular waste build up over time. Aging also results in an increased inefficency of certain cellular machinery which must also be disposed of. It has been documented that accumulation of both oxidative damge and impairment of the cellular waste clearing system contribute to the pathogeneiss of AMD and many other age associated diseases (49). Animal models which have knockout of genes assoicated with the visual cycle such as Abca4 accumulate atRAL and exhibited dysfuctinoal photoreceptor and RPE cells(49). These animals had characteristic yellowish drusen deposits extracellularlly and intracellular lysosomal lipofuscin, Light exposure in these animals also increases oxidase -mediated overprocution of intercellular ROS (49). It is therefore evident that the abnormal accumulation of byproducts of the visual cycle can lead to retinal degnenration. Another by-product of the visual cycel which can induce damage within the retina is N – retinylidene – N-rtylethanolamine (A2E). A2E accumulation with the specialised RPE cells leade to DNA damage and ultimately retinal degeneration. A2E has also been shown to accumulate in aging RPE cells and increase the accumulation of certain inflammatory molecules (49).

Due to the constant exposure of visible light the retina is highly susceptible to oxidative damage. As mentioned earlier, smoking can increase the likelihood of development of AMD because of the increased ROS production in the retina. It has been demonstrated that high doses of antioxidants can reduce the progression of intermediate and late stage AMD (50). Antioxidants such as vitamin C, vitamin E increase the activity of SOD and catalase (50). This increase in oxidative stress in addition to build up of cellular waste can all contribute to the pathogenesis of AMD. Genetic mutations in genes associated with complementation are another important factor in AMD pathogenesis, as drusen has been found to contain certain complementation proteins (49). The complementation system enhances the immune systems functions by recruiting phagocytic cells to clear cellular debris. Impairment of the cell's complementation system thus can

impede efficient clearance of visual cycle waste and other cellular debris also increasing the likelihood of oxidative stress all of which can increase susceptibility to AMD (50). A large portion of the heritability of AMD is associated with immune system genes. Specifically, genes encoding complement components such as complement factor H (CFH) Y402H (Tyr402His) variant which is the most known genetic risk factor for AMD (51). This variant is a risk factor associated with the Caucasians susceptibility to AMD (51). AMD is also been associated with other complement factor genes like CFB (51). It has been reported that AMD – associated neovascularization is enhanced with increased expression of CFB in RPE with increased exposure to photoreceptor outer segment (POS) and pro inflammatory cytokines TNF- α and IFN- γ (51). Other evidence which supports the association of complement system in AMD is the use of CFB genes as protection against AMD (51).

Inflammation is the cell's natural response to factors which may compromise homeostasis such as pathogens and damaged materials or organelles. Pattern -recognition receptors (PPR) such as Toll- like receptors (TLR) complement components and inflammasomes all participate in recognizing threats or damage associated molecules patterns (51). NLRP3 is activated in the RPE by components found in drusen such as A2E (N- retinylidene- N – retinylethanolamine), amyloid – β. NLRP3 inflammasomes in RPE leads to accumulation of HNE (4 – hydroxynonenal) and intracellular protein aggregates which contribute to dysfunctional autophagy (51). Drusen has been found to contain many inflammation related factors along with other damaging components described previously and is thought to be an inflammatory node in AMD pathogenesis (51). There is also production of recruitment of leukocytes and dendritic cells and microglia cells (52).

One major component of age assoicated diseases is the delcine of autophagy and the cell's waste disposal systems. AMD is a complex age associated disease which involves, oxidative stress, inflammation and impaired autophagy. Autophagy is an imporant cellular mechanisms designed to rid the cell of damaged organelles, protein

aggegrates. As discussed previously, by-products of the visual cycle our inefficienctly cleared, build up over time and contirbutie to oxidative stress and inflammation all features of AMD. It has been shown that oxidative stress directly impairs autophagy in RPE cells.

RETINAL PIGMENT EPITHELIUM

RPE cells are highly specialized pigmented cells that are essential to retinal homeostasis and functionality. RPE are hexagonal in shape and are highly polarized. RPE cells are connected by tight junctions and are essential for maintaining the blood retinal barrier in order to protect the eyes from pathogens that circulate in the blood and allows for strict regulation of immune system components within the retina (6, 7,50). RPE cells also produce immunosuppressive factors to help downregulate T-cell activity as well as other complement proteins (50). Another function of RPE is the transportation of fluid and nutrients to the retina. RPE cells also help to maintain fluid, ion and ph balance in the sub-retinal space. RPE participates in the visual cycle by absorbing stray light and recycling retinols within melanosomes (6). Melanosomes within the RPE also function as scavengers for reactive oxygen species and harbor metal ions like iron (53). Melanosomes are typically found on apically in the RPE cell. With age, melanosomes become more basally located and even can be found fused with lipofuscin granules as melanolipofuscin complexes (53). RPE secretes Vascular Endothelial Growth Factor (VEGF) for maintenance of choriocapillaris and produces Pigment Epithelial Derived Factor (PEDF) to suppress pathological angiogenesis (Figure 1.4, 8). On the apical surface, RPE microvilli surround the tips of rods and cone photoreceptors, which facilitates the phagocytosis of photoreceptor outer segments (POS) (Figure 1.4; 9, 10). Daily phagocytosis of shed photoreceptor outer segments is key for maintaining retinal functionality. Each RPE cell associates with approximately 20 photoreceptor cells and is responsible for the phagocytosis and degradation of more than 100 million POS discs over the lifetime of the individual. RPE is post-mitotic and subject to numerous stresses such as heat stress associated with absorption of light, photo toxicity and oxidative damage. With age and accumulated oxidative stress, the clearance efficiency decreases in RPE. Such a reduction along with other age-associated changes in the RPE and Bruch's membranes, are central

to the development of AMD (1, 2). RPE degeneration is the first event which occurs in AMD (52). The loss of the RPE in AMD pathogenesis leads to and increase in metabolic burden for nearby cells like photoreceptors (52). RPE is highly metabolically active and contains a large concentration of polyunsaturated fatty acids, it is therefore not surprising that it can be susceptible to oxidative stress (51). In addition to the constant exposure to light RPE also consume large amounts of oxygen both of increases these cell's susceptibility to chronic oxidative stress. RPE harvested from AMD donors produce more ROS and exhibited increased susceptibility to oxidative stress (49). The RPE thus is the primary site of injury in AMD. Within the macula region of the retina, there is an accumulation of oxidatively damages molecules which serve as a source for chronic oxidative stress in RPE (52). Molecules such as carboxyethylpyrrrole, malondialdehyde, 4 – hydroxynoneal are accumulated within the macula (52). There is also an age associated increase in accumulation of lipofuscin in the lysosomes of RPE (52).

As AMD progresses many of the pivotal support functions provided to the neighboring photoreceptor cells by the RPE cells are halted. Photoreceptors cells and RPE cells are eventually lost in late stage AMD.

PHOTORECEPTOR OUTER SEGMENT RENEWAL

The outer segment of photoreceptors is composed of densely packed lipid rich discs. POS renewal is a lifelong process, whereby new membrane disks are formed at the proximal end of the outer segment while there is a diurnal shedding of the distal spent outer segment (11, 12). In a series of pulse chase experiments with radioactive amino acids, Young and colleagues showed bands of radioactive material moving from the basal end of the photoreceptor outer segment to the distal end over time (Figure 1.5). The experiments suggested that photoreceptor outer segment discs are constantly being produced on the proximal end of the outer segment and shed at the distal tip (12, 13; Figure 1.5). Once the photoreceptors have shed the distal tip of the outer segment, RPE cells phagocytize the shed outer segment. Clearance of POS prevents accumulation of photo-oxidative compounds and helps recycle of visual pigments. POS turnover has been shown to be essential for survival of both the photoreceptor cells and RPE. Ingested DHA, a byproduct during the photoreceptor renewal process and subsequent phagocytotic stage, is synthesized to neuroprotectin 1 in RPE (53). Neuroprotectin 1 is a substance which provides protection from oxidative insult (53). Failure in POS turnover in humans and rodents has been reported to induce retinal degeneration (10). Debris and by- products from POS phagocytosis is one major source of oxidative stress in RPE (50). Inefficient clearance of the ingested POS can lead to accumulation of lipid deposits inside and outside of the RPE. Drusen deposits observed in AMD are the formation of undigested POS outside the RPE. Lipofuscin are accumulated abnormal deposits within the RPE. Dysfunction in degradation and clearance of POS results in the abnormal accumulation of lipid and protein aggregates as a result of oxidation of unsaturated fatty acids (49). Lipofuscin contains proteins, lipids and saccharides (49). Both drusen and lipofuscin can lead to degeneration of the RPE and photoreceptors (37). Undigested POS not only initiates oxidative stress in the RPE but also confounds inflammation. This confound between inflammation of

oxidative stress in the RPE is thought to be an important association in the pathogenesis of AMD. It has been reported that oxidative tress can regulate expression of both CFH and CFB and that phagocytized oxidized POS prevents the synthesis and secretion of CFH in RPE cells (51). In fact, it has been shown that oxidative stress causes the RPE cell to be vulnerable to complement – mediated injury (51).

PHOTORECEPTOR OUTER SEGMENT PHAGOCYTOSIS

Phagocytosis by RPE belongs to a class of non-inflammatory clearance used to remove cellular debris and apoptotic cells (11). The phagocytic pathways are believed to be mechanistically similar, however RPE clearance of POS occurs daily. RPE cells and POS are in constant contact, whereas macrophages are activated in response to inflammation and cell death (14). Some of the elements of phagocytosis of POS by RPE are similar to inflammatory phagocytic pathways. However, it is important to consider some unique features of phagocytosis by RPE. RPE cells, unlike other cell types that actively phagocytize, are post-mitotic and phagocytose and degrade about 10% of the total photoreceptor volume daily (13). RPE are the most active phagocytic cells in the human body and any inefficiency builds up over a lifetime (15). Thus, recognition and phagocytosis of POS by RPE is likely a highly regulated phenomenon at a molecular level, which likely involves many proteins (14). With age, the ability of the RPE to handle the POS influx, likely decreases, causing undigested lipid rich POS to accumulate in the RPE and form intracellular and extracellular deposits, called drusen, which are the hallmark of AMD. Understanding the unique form of phagocytic activity of RPE involved in the clearance and degradation of POS is crucial for finding treatment strategies against AMD. Impaired lysosomal clearance also decreases phagocytosis in RPE.

The stages of RPE phagocytosis include recognition and attachment of POS, internalization of POS, formation of the phagosome, fusion with lysosome and degradation (1). Some of the molecular mechanisms and proteins involved with each stage have been identified as depicted in Figure 1.6. Most of our knowledge concerning phagocytosis of POS by RPE is concentrated to factors involved in the recognition and internalization of POS. For RPE cells to recognize POS and initiate engulfment POS must contain a signal recognized by RPE cells. Similar to apoptotic cells, it has been shown that POS display

phosphatidylserine “eat me’ signal. Normal RPE cells recognize and bind to phosphatidylserine-containing liposomes (14). Maximum phagocytosis occurs when the lights are on during awake cycles and the binding of POS to RPE apical membrane demonstrates a diurnal pattern attributed to differences in the levels of integrin receptor, αVβ5. The integrin receptor selectively binds POS, but not latex beads (4). Animal models deficient in the specific integrin receptors display loss of circadian rhythm of phagocytosis and demonstrate age related accumulation of lipofuscin, or lipid containing residues of lysosomal digestion (14). The binding of shed POS to αVβ5 integrin receptors on RPE cells is mediated by a ligand, milk fat globule EGF-factor 8 (MFG-E8), which is secreted by RPE cells on recognition of the αVβ5 RGD motif (14). MFG-E8 deficient mice display similar phenotypes as that observed in αVβ5 deficient mice, however MFG-E8 mice do not display age related blindness (14). After POS is attached to the RPE membrane, focal adhesion kinase (FAK) co-localizes with αVβ5, and FAK becomes activated through tyrosine phosphorylation (14). Activated FAK phosphorylates Mer tyrosine kinase (MerTK) resulting in the internalization of POS (16). Inhibiting FAK results in blocking of internalization of POS but does not affect αVβ5 binding of POS to RPE. A naturally occurring deletion in Royal College of Surgeon (RCS) rat results in a dysfunctional MerTK protein. Binding occurs but internalization of POS is inhibited in RPE of these rats. In RCS rats, disruption of POS phagocytosis leads to accumulation of debris within sub retinal space, which lead to degeneration of photoreceptors and blindness (4). This leads to an accumulation of shed but not phagocytosed POS in the sub-retinal space, rapid photo receptor loss and eventual blindness (17).

Age – related stress to the RPE can also lead to inefficiency of the clearance pathway. RPE cells are post mitotic and are not renewed over a lifetime. Accumulation of intercellular debris can lead to dysfunction in the RPE. In AMD, there is an extracellular accumulation of fatty protein deposit which is a clinical feature of the disease. Disruption in RPE’s ability to bind and degrade POS leads to rapid retinal degeneration. Therefore, a

molecular dissection of factors involved in phagocytosis of POS by RPE and trafficking of ingested POS to lysosome for degradation could lead to new targets for the treatment of retinal degenerative diseases like AMD.

After the initial stages of recognition and internalization in POS phagocytosis, less is known about the final stages of phagosome maturation (Figure 1.6). Phagocytosis has been more well studied in other cell types such as macrophages. The proteins involved in the phagosome maturation process in these cells have been identified. It has been reported that some of these proteins are expressed in rat RPE, suggesting that similar phagosome maturation pathway maybe involved in RPE (52). The precise similarities between macrophage phagocytosis and RPE phagocytosis has not yet been reported. The goal of this project is to uncover regulators that have not been previously identified to be involved in RPE phagocytosis process.

Lysosomal clearance can be disrupted in various way in the RPE during AMD disease progression. Lysosomes are the endpoint for various cellular clearance mechanisms including, phagocytosis, autophagy and endocytosis. Lysosomes utilize cathepsins to degrade proteins. In the RPE Cathepsin CTSD has the main responsibility of degrading POS and rhodopsin glycopeptide in RPE lysosomes (52). CTSD deficient mice have been shown to develop retinal degeneration (52). Inhibitors of lysosomal cysteine proteases are enriched in RPE cells (52).

Increased accumulation of oxidized low-density lipoproteins, internalized POS reduce the degradation of newly internalized POS and increase cellular and oxidative stress in RPE (52). Once formed, it is believed that lipofuscin build up within the cell cannot be degraded via proteasomal or lysosomal enzymes and is transported outside the cell via exocytosis (52).

Lysosomal pH is another factor which determines clearance of internalized POS. Elevated lysosomal pH is observed in RPE from retinal degeneration model and human ARPE-19 cells exposed to A2E (52)

Internalized but not cleared POS causes intense cellular and oxidative stress within the RPE ultimately leading to retinal degeneration and AMD pathology.

LIMITATIONS OF CURRENT METHODS FOR STUDYING PHAGOCYTOSIS OF POS BY RPE

In order to begin to elucidate other molecules that are involved in phagocytosis of POS by RPE there must be a quantifiable method for assaying the dynamics of the pathway. The lack of a standardized or well quantifiable method has made it difficult to make new discoveries in this area. Conventional tools mainly focus on immuno-detection of components of degraded POS in RPE (10,38). This only allows description of dynamics of POS binding and/or internalization and degradation without information relating to the intermediate process of POS trafficking to lysosomes. In our laboratory, we utilize the immunodetection of RPE cells with rhodopsin antibody which measures phagocytic load and kinetics of phagocytosis via western blots (18, 38). The outer segment of photoreceptors contains photon-capturing rhodopsin as depicted in Figure 1.7 A (19). Rhodopsin is not expressed by RPE cells. Upon engulfment of POS, RPE cells become positive for rhodopsin. Over time, as the cells begin to degrade POS and slow down uptake, rhodopsin levels also decrease. After challenging RPE with POS the cells are washed and harvested for immunodetection with rhodopsin antibody. Levels of rhodopsin gradually decrease with time as RPE cells degrade the ingested POS (Figure 1.7). The use of rhodopsin immunodetection provides a global assessment of the RPE cell's ability to uptake and degrade POS. This method does not allow the dissection of the underlying molecular mechanisms of specific stages of phagocytosis, namely the trafficking stage. Thus, the conventional methods of analysis only allow the study of mass quantitative changes but are not sensitive enough for dissecting molecular mechanisms of phagocytosis. There is thus an unmet need for a quantifiable method to assess specific stages of phagocytosis of POS. A major goal of this project was to develop a quantifiable fluorescent assay for assessing the trafficking stage of phagocytosis of POS within RPE.

FOSTER RESONANCE ENERGY TRANSFER

To develop the fluorescence assay for monitoring of photoreceptor outer segment trafficking in RPE, we used Foster Resonance Energy Transfer or FRET. FRET is an energy transfer mechanism between two fluorophores that are in close spatial proximity to each other; typically, between 10 to 400nm apart, as diagrammatically presented in Figure 1.8. (20). There must be significant overlaps between emission spectra of the donor and excitation spectra of the acceptor emission spectra for the pair to be compatible for FRET. When the donor molecule is excited, and emits energy, it is transferred to a nearby acceptor molecule (Figure 1.8) (20). The distance between the donor and acceptor fluorophores determines the efficiency of the energy transfer. The intensity of the FRET fluorescence emitted by the acceptor fluorophore reflects the co-localization of the two fluorophores. When labeling targets of interests with FRET capable donor and acceptor fluorophores, FRET fluorescence can be measured and used as the indicator of the co-localization of the targets. The technique has been widely used to measure co-localizations of proteins and organelles (21, 22).

Results

DETERMINATION OF FRET DONOR AND ACCEPTOR PAIR

To develop the FRET based assay for POS trafficking in RPE, we required fluorophores to label the ingested POS fragment and the lysosomes respectively. The first fluorophore serves as the donor and labels the membrane of POS. The second fluorophore which serves as the acceptor labels lysosomes in the RPE cell. The movement of the POS to the lysosome is then monitored. The experimental workflow of FRET experiments is depicted in Figure 1.9.

For selection of a FRET donor we began with the electron donor dye NBD or 7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl0-1,2-Dihexadecanoylsn-Glycero-3 Phosphoethanolamine, Triethylasmmonium Salt. NBD is a lipid dye that is used for labelling the membranes of lipids (20-22). The FRET acceptor used was Lysotracker DND 99, a red–fluorescent dye for labeling and tracking acidic organelles in cells (39). Lysotracker would in turn label the lysosomes in the RPE cells. As depicted in Figure 1.10 the pair satisfy the initial requirement for FRET donor and acceptor pair. NBD has an excitation peak at 463nm and an emission maximum at 536nm that has an overlap with Lysotracker's excitation spectra (Figure 1.10). Lysotracker emission peak of 590nm, is the energy transfer peak which will be monitored when using this donor and acceptor pair. To validate the use of NBD labelled POS and Lysotracker as FRET donor and acceptor pair, we first mixed equal molar concentrations of both dyes in solution and monitored for energy transfer using a spectrofluorometer. With excitation at 463nm, characteristic excitation of NBD, we observed an energy transfer peak at 590nm, characteristic emission of lysotracker (Figure 1.11). These preliminary experiments validate the use of NBD labeled POS and lysotracker as a FRET donor and acceptor pair.

Next, we validated NBD labeling of POS and use in our immortalized human RPE cell line, ARPE-19. POS were isolated from pig retinas using established discontinuous

sucrose gradient protocol (31-32). After isolation POS were labeled with NBD by mixing. NBD attachment to POS was validated via immunofluorescence staining. NBD labelled POS were identified in ARPE-19 cells following incubation with fluorescence excitation at 488nm (Figure 1.12). ARPE-19 cells recognize and uptake NBD labelled POS. Following lysosome labelling of ARPE-19 cells with lysotracker, fluorescence imaging showed co-localization of NBD labelled POS and lysotracker labelled lysosomes. This experiment validated NBD labelling of POS and the ability of ARPE-19 cells to recognize the labelled fragment.

ARPE-19 cells seeded on 96 -well plates cells were incubated with NBD - POS for two hours. After incubation with NBD - POS, cells were washed and then incubated with lysotracker for thirty minutes to label lysosomes. Using a plate reader, an emission curve was collected when cells were excited at the excitation wavelength of NBD (463nm). With increasing concentrations of NBD - POS, we observed an increase in intensity at the characteristic emission maximum for lysotracker, corresponding to an energy transfer peak (Figure 1.13).

The next FRET donor and acceptor pair designed to assay POS trafficking in RPE cells were FITC labelled POS and Lysotracker. FITC labelling of POS has been used in the field for fluorescent recognition of POS in RPE (11,18). Immortalized human RPE cell lines like ARPE-19 recognize and uptake FITC labelled POS. These current established protocols for FITC labelling of POS result in high background and promote aggregation of the labelling dye. We sought to first optimize the current protocol and utilize FITC labelled POS as a FRET donor. Using several different conditions including alerting FITC concentrations, wash and mixing times we developed a protocol to optimize FITC labelling of POS, reducing background and aggregation. Figure 1.17 B shows optimized labeling of FITC POS labelling with reduced aggregation of dye.

Figure 1.14 depicts the overlap of FITC excitation (donor) and lysotracker emission(acceptor), the satisfaction of criteria for FRET donor and acceptor pair. To

validate the donor and acceptor pair, using a spectrofluorometer, we mixed equal molar concentrations of FITC labelled POS and lysotracker. We observed an energy transfer peak characteristic of lysotracker emission maximum when the mixture was excited at 490nm, excitation maximum of FITC (Figure 1.15). This experiment verified the feasibility of FITC - POS and Lysotracker as a FRET donor and acceptor pair.

Finally, to ensure labelling of POS does not alter conformation of POS fragments we performed immunoblotting experiments of naïve vs NBD - POS and FITC- POS. Figure 16 shows bands observed when samples were probed with rhodopsin. NBD - POS aggregates at the top of the membrane. This corresponds to aggregation of NBD labelled POS. POS dimer and monomers can be seen in naïve and FITC-POS but not in NBD – POS. FITC- POS monomers and dimers maintain the same molecular weight as naïve POS (Figure 1.16). NBD labelling of POS promotes aggregation of POS fragments. We determined this phenomenon would not be favorable in a trafficking assay. Thus, FRET experiments were carried out with FITC – POS as FRET donor and Lysotracker as FRET acceptor.

UPTAKE OF FITC- POS CAUSED DOSE –DEPENDENT INCREASE IN FRET INTENSITY

FITC – POS were incubated with RPE cells for 2 hours. Unbound POS was removed by washing and cells were incubated with lysotracker to label the lysosome (Figure 1.18). Using fluorescence microscopy, we observed RPE uptake of FITC - POS and trafficking of FITC - POS to the lysotracker labeled lysosome. Time lapse images in Figure 1.18 show the movement of FITC – POS (A – C) and fusion to lysotracker labelled lysosome corresponding to signal in FRET channel (C – D). The FRET channel was setup with excitation at 490nm (donor excitation) and emission collected at 590nm (acceptor emission). FRET intensity in the FRET channel was calculated using Image J. All images were captured at the same settings keeping exposure times the same. Intensity in FRET

channel increased as FITC – POS concentration increased (Figure 1.19). 25ul of FITC-POS resulted in highest FRET intensity observed in the FRET channel.

UPTAKE OF FITC – POS CAUSED TIME – DEPENDENT INCREASE IN FRET ACTIVITY

RPE cells were incubated with FITC – POS at varying time points (1h – 4h) (Figure 1.20). Cells were then washed to remove attached but not engulfed FITC - POS. Cells were then incubated with lysotracker to label the lysosomes. After incubation with lysotracker, cells are live imaged. As done in previous acquisitions, images were captured at the same setting; exposure times were the same across experiments. We observed a time dependent increase in FRET activity as FITC - POS is up taken (Figure 1.20). FRET channel was set up as previously, with excitation at 490nm and emission at 590nm. FRET intensity was calculated using Image J software. FRET intensity values increased with time with a peak intensity between 2h and 3h time point. Figure 1.20 show representative image from one experiment. Our developed method can detect small physiological changes of POS trafficking to lysosomes. The increase in FRET intensity values over time correspond to POS trafficking to the lysosome. FITC- POS intensity values (Figure 1.21 C) follow a trend comparable to traditional western blot assays with peak intensity value at 3h. The 3h peak corresponds to the peak time required for RPE to uptake POS. The FRET assay's peak intensity at 2h corresponds to the peak time required for POS and lysosomal fusion. This observed difference in peak intensity of FRET and FITC-POS distinguishes the FRET assay from the conventional assay. Thus, the developed FRET assay measures trafficking and not uptake of FITC- POS in RPE.

SPECTRAL BLEED THROUGH CORRECTION

As a result of spectral overlap between donor and acceptor there will always be contamination in the FRET channel (40,41). Signals will bleed through the FRET channel that are not a direct result of an energy transfer but rather noise from direct excitation of the donor by the acceptor's excitation energy. Emission of the donor in the acceptor channel and the excitation of the acceptor using the donor excitation wavelength will both contribute to spectral bleed through and noise in the FRET channel. To account for the contamination in the FRET channel certain corrections must be made (40,41). FRET efficiency can be calculated by normalization for difference in the donor and acceptor expression levels as well as normalization of the bleed through. We used the following equation and FRET and Colocalization Analyzer on Image J to calculate the corrected FRET. Figure 1.22 shows the $FRET^{index}$ image derived from the Image J plug- in from a single experiment. This collected FRET index further validated our FRET experiments.

$$\textbf{FRET index image} = I_{\textbf{FRET}} - (\alpha_{\textbf{D}} I_{\textbf{Donor}}) - (\alpha_{\textbf{A}} I_{\textbf{Acceptor}})$$

VALIDATION OF FRET ASSAY

The second part of the development of this assay was to validate the asaay with known molecules involved in POS phagocytosis. We hypothesize that our assay gives distinct information regarding individual steps of RPE phagocytosis. Table 1 describes the expected outcomes of the validation experiments. In these experiments we impaired a specific step in the phagocytic process and observed if our assay can distinguish between the individual steps. We focused on the trafficking and degradation step. To impair degradation, we used chloroquine treatment of RPE cells. An impaired trafficking by knockdown atg5 in RPE cells.

CHLOROQUINE ENHANCED FRET SIGNAL DERIVED FROM UPTAKE OF FITC- POS

Chloroquine is known to increase lysosomal pH by accumulating within lysosomes as a deprotonated weak base. Treatment with chloroquine has been shown to lead to lysosomal dysfunction and RPE degradation in various animal models. Our laboratory has shown that treatment of ARPE-19 cells with chloroquine disrupts RPE phagocytosis (38). We hypothesized that impairment of POS degradation in RPE with chloroquine would result in increased FRET signal. RPE cells were incubated overnight with 20uM Chloroquine. On the day of the experiment, cells were challenged with FITC-POS for 2 hours. Unattached FITC-POS was washed off and cells were incubated with lysotracker containing media to label lysosomes. After lysotracker incubation cells were live imaged. Figure 1.23 shows representative images from a single experiment. Chloroquine treatment enhanced FRET intensity signal derived from uptake of FITC – POS (Figure 1.23). We also observed that chloroquine treatment enhanced the time dependent increase in the FRET intensity signal derived from uptake of FITC-POS. These results support our hypothesis that the developed FRET assay can offer useful information regarding molecules involved in the degradation stage of POS phagocytosis. Again, our assay is sensitive enough to detect physiological changes in POS phagocytosis. Impairment of degradation leads to enhanced FRET signal in our assay.

ATG5 KNOCKDOWN REDUCES FRET SIGNAL DERIVED FROM UPTAKE OF FITC- POS

Next, we validated our assay with Atg5 knockdown in RPE cells. Atg5 is an autophagy related gene involved in a non-canonical autophagy pathway which facilitates phagosome formation and POS degradation (42). Trafficking of POS but not uptake has been shown to be affected in Atg5 deficient cells (42). Phagosomes require Atg5 to move through the RPE and enter lysosomal compartment for degradation. We used Atg5

knockdown to impair the trafficking stage of POS phagocytosis. We hypothesized that Atg5 knockdown in RPE cells will exhibit reduced FRET signal in our assay. We used siRNA to knockdown Atg5 in RPE cells (Figure 1.24). RPE cells were then challenged with FITC - POS as previously done. Atg5 knockdown in RPE reduces FRET signal derived from uptake of FITC - POS (Figure 1.24). FITC- POS uptake was not affected by Atg5 knockdown. FITC- POS intensity values depicted in Figure 1.25 correspond to RPE uptake of POS. In western blot assay atg5 knockdown cells uptake FITC – POS although they lack the characteristic uptake peak at 2h observed in scrambled RPE cells (Figure 1.25 C & D). Our developed FRET assay is sensitive enough to detect physiological changes in trafficking of POS in RPE.

Discussion

RPE clearance of POS is an essential process which contributes to overall retinal homeostasis. Age related stresses can lead to inefficient clearance of shed POS. This inefficient clearance of POS results in accumulation of intracellular and extracellular debris, a classic hallmark of some retinal degenerative disease. Most knowledge relating to the unique phagocytic pathway relates to the molecular machinery involved in recognition, internalization and degradation of the ingested material. Limited methods for studying the intermediate step creates this bottleneck thereby impeding are ability to understand the molecular regulators involved in POS trafficking to lysosomes. We have developed a novel method to detect the smallest physiological changes in POS phagocytosis in RPE. We have characterized and validated the novel FRET assay to detect trafficking changes as well as degradation changes in RPE. We have also optimized FITC labelling of POS. With the optimized FITC- POS we developed a method to observe and quantify POS trafficking in RPE using FRET. The FRET assay offers sensitivity to observe even the smallest physiological changes. Conventional assays lack sensitivity to distinguish changes within specific steps in the phagocytosis of POS. The sensitivity afforded by this assay is useful for deciphering underlying mechanisms of POS trafficking. The developed assay can be used to explore candidate genes' role in specific stages of POS phagocytosis.

Future studies can focus on optimization of the assay for flow cytometry. FRET has been successfully used in fluorescence activated cell sorting assays (33 - 36). A flow cytometric approach to the current assay could be used for high throughput screens of candidate genes. We have carried out preliminary experiments using flow cytometry. Preliminary data shows that the assay could be useful for such experiments with further optimization.

Furthermore, this assay can be utilized in other systems to study the movement of molecules from one cellular compartment to another within the cell. By optimizing the developed assay with other probes tracking other organelles the same phenomena can be used to monitor endosomal fusion in other models. The novel assay provides a foundation to be adapted in other systems.

One major shortcoming of the developed assay is that it easily underestimates the true quantitative value of the FRET signal. In other words, the FRET signal detected in our assays maybe quenched prematurely underestimating the amount of POS phagosomes which have fused with lysosomes. Also considering the heterogenous nature of lysosomes as well as POS particles labelling of both donor and acceptor would also produce a heterogenous mixture of donor and acceptor which could affect energy transfer. The FRET signals produced in these experiments although would generally produce information regarding phagosome fusion with lysosome, would be less reliable for estimating uniformity of the FRET signal.

Chapter 2 Materials and Methods

CELL CULTURE

ARPE- 19 cells (American Type Culture Collection) were grown and passaged in DMEM/Ham's F12 50/50 mix (Mediatech) and supplemented with 10% FBS (Sigma Aldrich) (38). For FRET experiments cells were grown in 27mm glass bottom plates (Nunc^{TM}). For siRNA transfection cells were passaged at 90% to 100% confluence.

SMALL INTERFERING RNA (SIRNA)

siRNA were synthesized by Integrated DNA Technologies. The targeting sequences are as follows:(VPS11#1) CAG CUC AUC CUG UGC AAU, CCA GCA GAU UGC ACA GGA (VPS11#2) CAA AGA AGU GAG UGG UCU, GCA GGA ACC ACU CAC (VPS18#1) AGG CUC AUG CAC AGC UGA, CCA GGA AUC AGC UGU GCA (VPS18#2) GAA UAC ACU ACC UGA, GGA GGU GUC AGG UGC GA (VPS18 #3) GGG AUG UGA CGA CGG AUG AG, CAC CCA UCC UCA UCC GUC (ATG5#1) ACG CUA AAA GGC UUA CAG, UCU GAU ACU GUA AGC CUU (ATG5 #2) UGU UUC ACG CUA UAU CAG, UCU CAU CCU GAU AUA GCA (ATG5 #3) CUG AAA GAC CUU UCA UUC, AGC UUC UGA AUG AAA GGU. Scrambled siRNA was obtained from Life Technologies. Cells were transfected with 100pmole of siRNA by lipofectamine (Lipofectamine 200). Forty – eight hours after transfection cells were used for functional analyses.

POS PHAGOCYTOSIS ASSAY

POS were harvested from fresh porcine eyes obtained from slaughterhouse as previously done (38). Cells were challenged with POS at 10 :1 POS: RPE ratio. POS were added to the culture and incubated at specific times After incubation period, cells were washed with HBSS to remove unbound POS. Downstream assays were then carried out. For western blot assay cells were harvested at specific time points. For FRET assay live imaging was conducted after washing.

WESTERN BLOT ANALYSIS

Cells were harvested and then lysed in buffer containing Cell Lytic ^{TM} Cell lysis Reagent (Sigma Aldrich), 2x Laemmli sample buffer (Bio-Rad) at 1:10 ratio and 10mM glycerophosphate, 10 mM pyrophosphate, 1mM NaF, 1 mM Na_3VO_4 , and protease inhibitor cocktails. Samples are then sonicated and resolved n SDS- PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with specific antibodies and signals were detected using Odyssey Infrared Imaging System (LI-COR).

ANTIBODIES AND CHEMICALS

Secondary antibodies were obtained from Life Technologies or LI-COR Biosciences. Chloroquine was obtained from Sigma – Aldrich. Lysotracker DND- 99 was obtained from Invitrogen. FlTC and NBD-PE were obtained from Thermo Fisher Scientific. Primary antibodies used in the study are listed in Table 2.

IMMUNOFLUORESCENCE MICROSCOPY

ARPE-19 cells were seeded on cover glass as previously described (38). For cells incubated with POS, POS was washed with HBSS as described previously. Cells were fixed with 4%

paraformaldehyde. Following fixation cells were permeabilized with cold methanol. Cells were then blocked in 10% (v/v) FBS/ 0.5%(v/v) Triton X – 100/1x Tris Buffer Saline (TBS). After blocking, cells were labelled with primary antibodies, followed by labelling in appropriate secondary antibodies. Nuclei were stained with DAPI. Samples were then mounted on glass slides with mounting medium (Electron Microscopy Service). Images were acquired with a Carl Zeiss LSM 510 confocal microscopy system or Carl Zeiss LSM 880 confocal microscopy system. Data were analyzed with Image J software or Zen software.

LIVE CELL IMAGING FRET ASSAY

ARPE- 19 cells were seeded in 27mm glass bottom plates or 4 chamber glass slides. Cells were then challenged with FITC – POS at a ratio of 10:1 FITC- POS: RPE at specific times. After incubation cells were washed with HBSS to remove unbound POS. Cells were then incubated with 400uM Lysotracker DND- 99 for 30 minutes to label lysosomes. After incubation lysotracker containing media was removed and replaced with HBSS for live imaging. Images were acquired with a Carl Zeiss LSM 510 confocal microscopy system or Carl Zeiss LSM 880 Live cell imaging system. Data were analyzed with Image J software (FRET & Colocalization Analyzer plugin) or Zen software

POS LABELLING

POS harvested from porcine eyes were mixed with either NBD or FITC. For NBD labelling, 2mM NBD was mixed with 100ul POS and placed on shaker for 1 hour protected from light. After incubation, POS was washed with HBSS and then resuspended in 100ul HBSS. For FITC labelling, 300ul POS, 40 ul of 1M $NaHCO_3$ and 3ul FITC were mixed

and placed on shaker for 1 hour protected from light. After incubation POS was washed with HBSS and then resuspended in 35ul of HBSS.

IMAGE ANALYSIS

All experiments were repeated three times (n=3). At least three images of random areas of interest were captured. All images were captured with uniform settings. Images were not modified prior to intensity analysis. Image J software (National Institute of Health, rsbweb.nih.gov/ij; version 1.51J) was used to measure intensity values in FRET channel and FITC channel in different images from different experiments. $FRET_{index}$ image was generated from Image J plug- in FRET and Co-localization analyzer.

STATISTICAL ANALYSES

All experiments were conducted in triplicates (n=3) and intensity values obtained from Image J were analyzed using GraphPad Prism seven software (GraphPad Software, Inc., LaJolla, Ca, USA). Between- group differences were analyzed by student's t – test or Mann- Whiney test and $P < .05$ was considered significant.

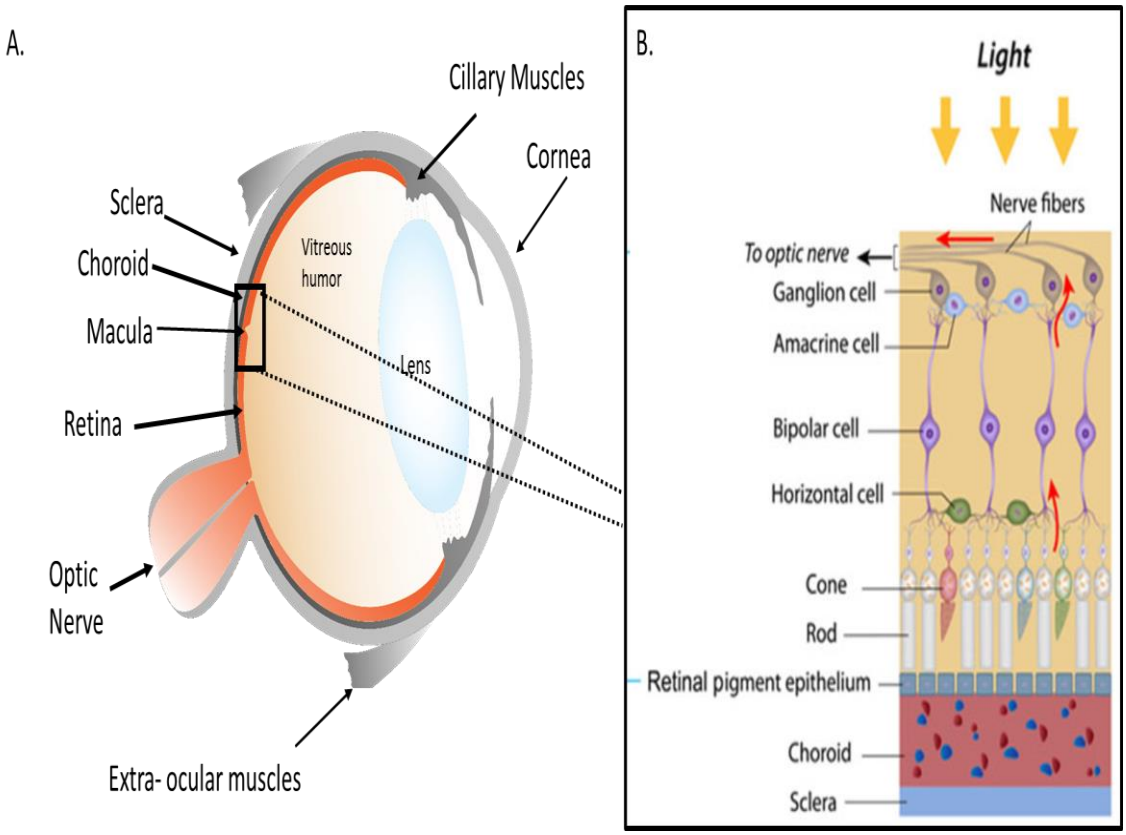

Figure 1.1 Representative image of human eye.

A. Sagittal section of human eye showing the distinct layers of tissue.

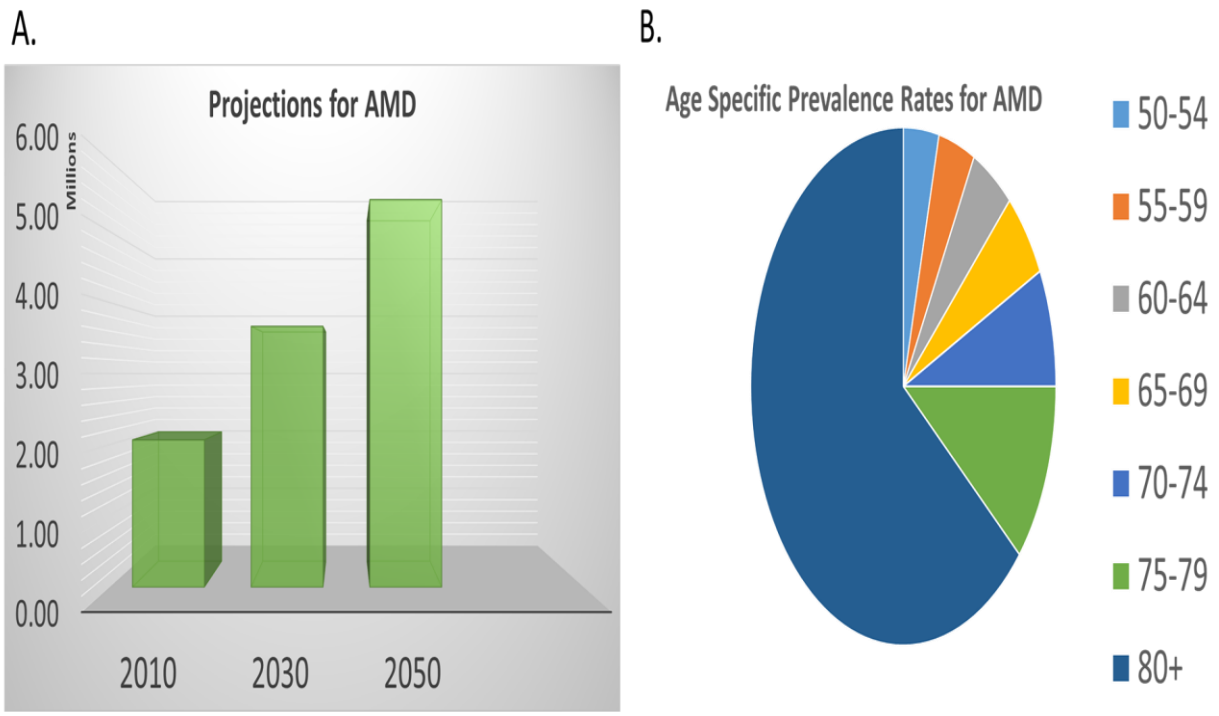

Figure 1. 2 Projection for AMD and Age Specific prevalence rates in 2010 in the United States.

A. Projections for AMD in millions. B. Age specific prevalence rates for AMD in 2010. AMD affects about 3.76% of people age 50 – 54, 3.91% of those 55- 59, 4.58% of those 60-64, 5.41% of people age 65-69, 7.34% of people age 70-74 , 11.25% of people age 75-79 and 63.75% of those 80 and older. From nei.nih.com)

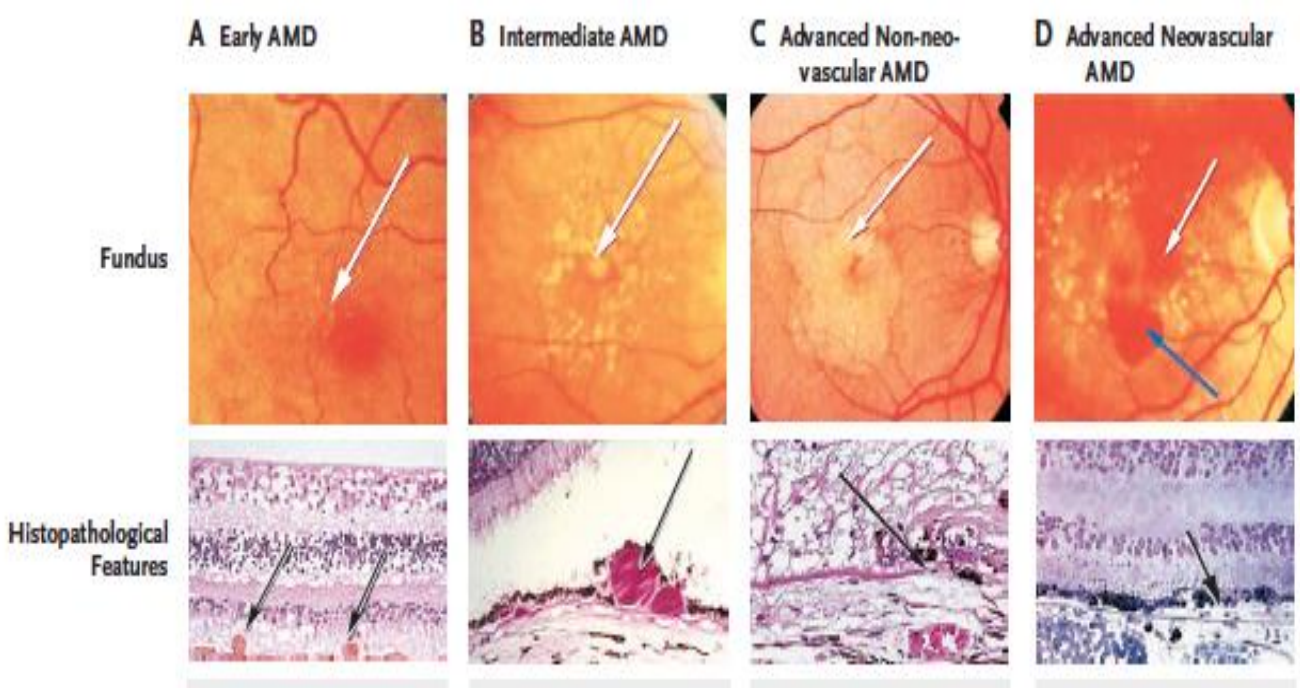

Figure 1. 3 Clinical Characterization of AMD

A. Photograph of fundus showing early AMD with white arrow indicating medium drusen deposit. Histopathological photograph showing drusen deposits (black arrow) between Bruch's membrane and RPE. B. Intermediate AMD with large drusen (white arrow) deposits with histopathological photograph of large drusen (black arrow). C. Geographic atrophy (white arrow) and Histopathological photograph showing geographic atrophy with loss of Bruch's membrane (black arrow. D. Photograph of fundus with neovascular AMD showing sub retinal hemorrhage (blue arrow) and choroidal neovascularization (white arrow). Histopathological photograph shows choroidal neovasculatiotion (black arrow). From Jager RD, Mieler WF, Miller JW., N Engl J Med. 2008

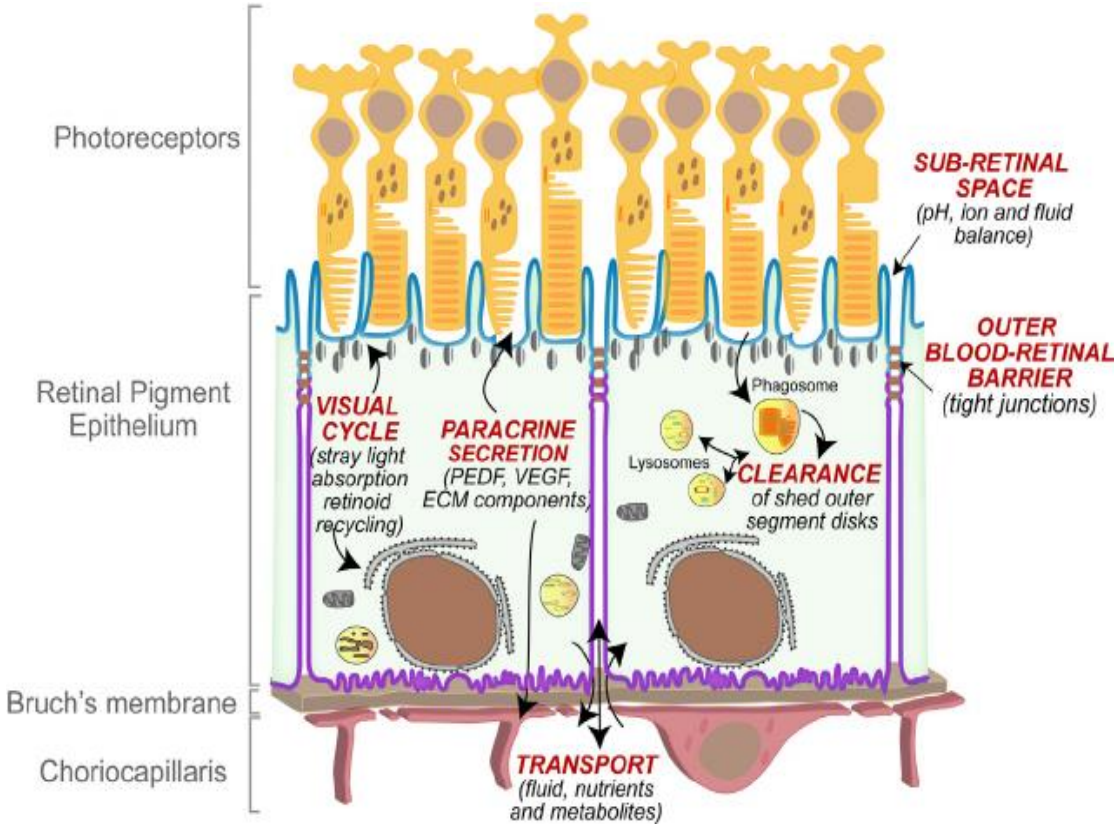

Figure 1. 4 Retinal Pigment Epithelium maintains retinal homeostasis and functionality

RPE cell makes up the outermost layer of the retina. RPE lay in between photoreceptors and Bruch's membrane. Bruch's membrane is a thin extracellular matrix which lays between the RPE and choroidal blood vessels. RPE is polarized with distinct apical (blue) and basolateral membrane (purple) domains which contain tight junctions and adhesions Melanosome (brown). RPE major functions are highlighted and described in the text. Adapted from Toops & Lakkaraju, Exp Eye Res, 2014

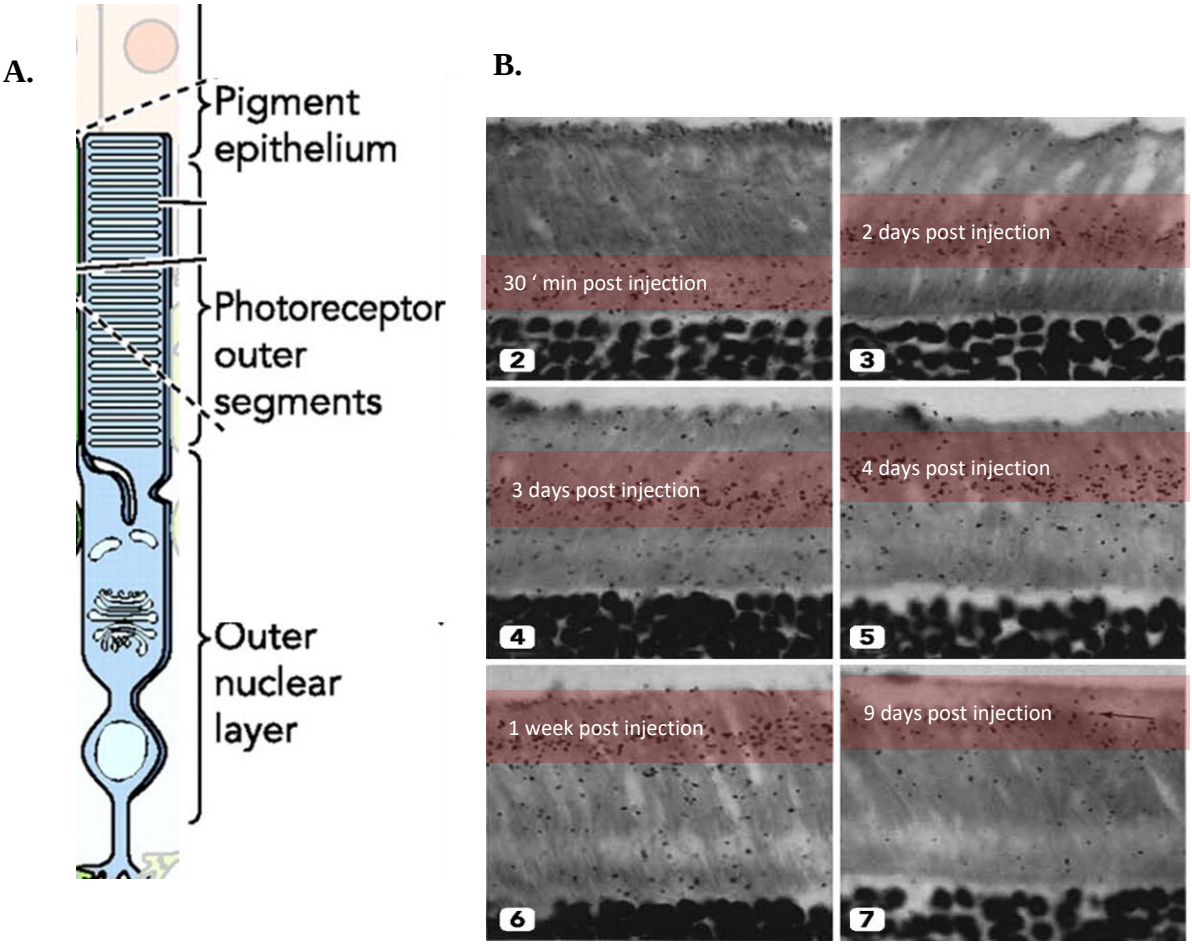

Figure 1. 5 Photoreceptor outer segment renewal

A. Radio autographs showing progression of radioactive protein band during renewal. Numbers indicate time after injection: 0.5h (2), 2 days (3), 3 days, (4), 4 days (5), 1 wk. (6) and 9 days (7). Arrow in 7 indicating a shed packet of outer segment. B. Representation of radioactive band progression during photoreceptor renewal. From Brian M. Kevany, and Krzysztof Palczewski Physiology, 2010 and Young , Journal of Cell Biology 1967.

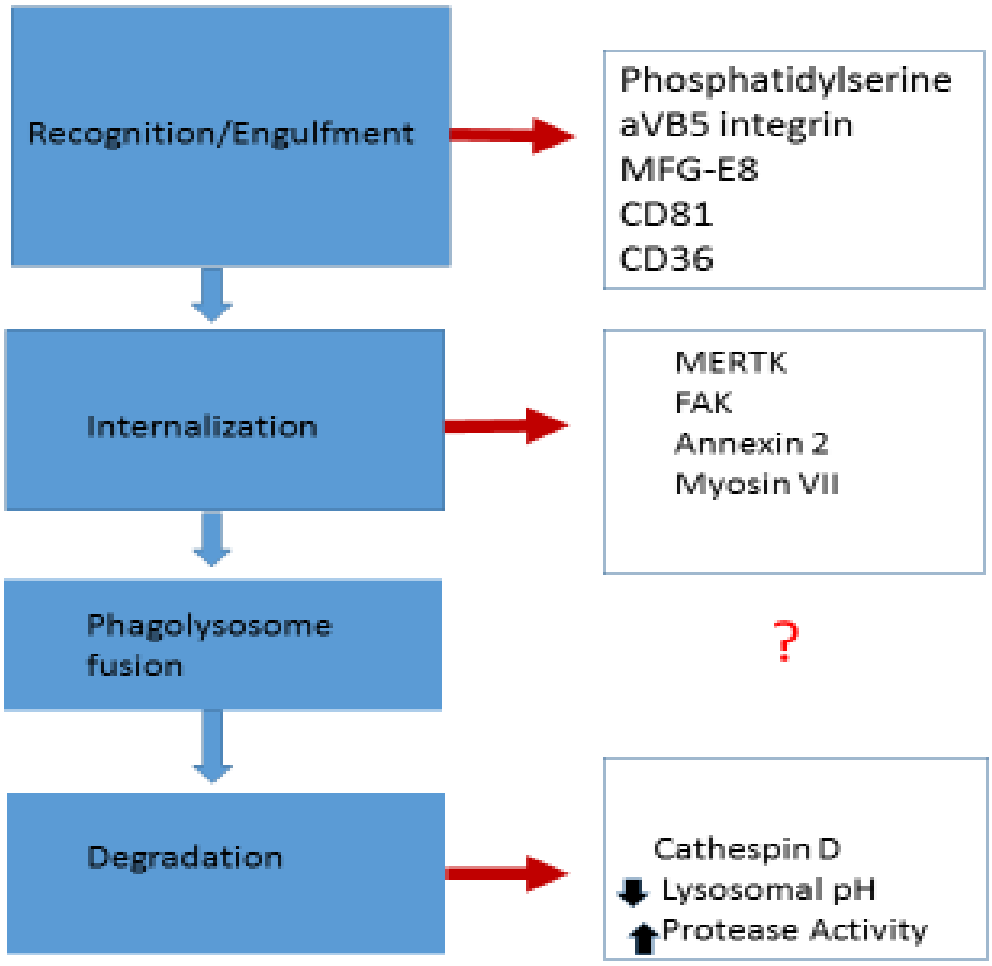

Figure 1. 6 Stages of POS phagocytosis with known regulators as described in text

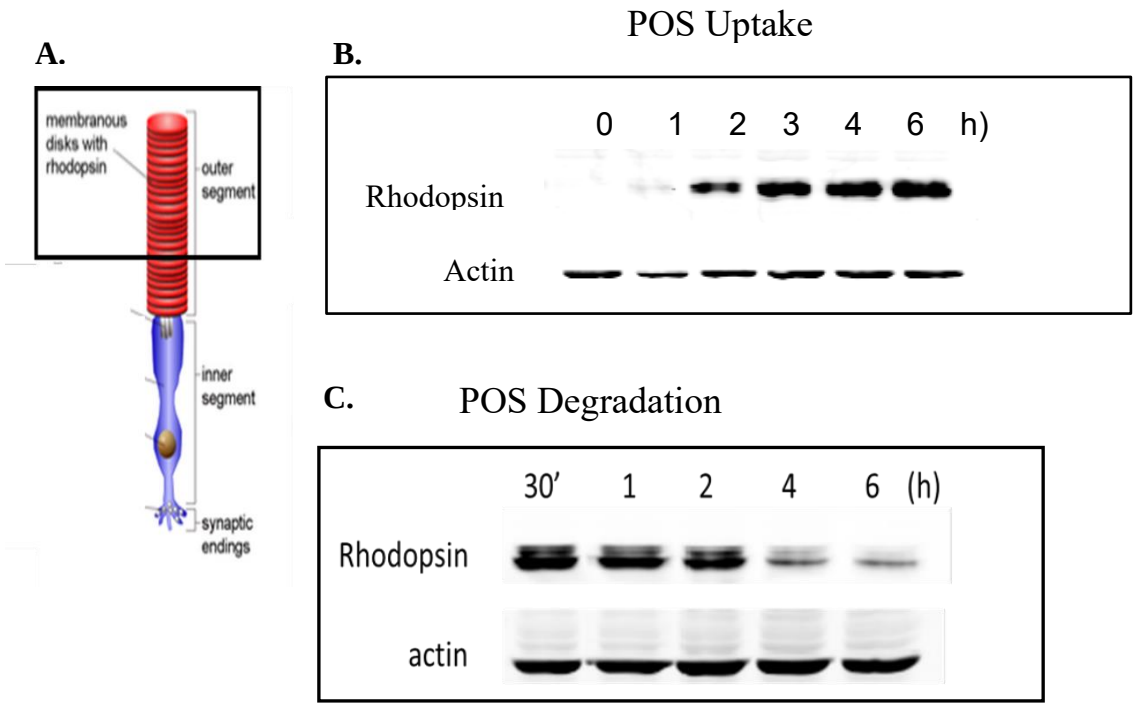

Figure 1. 7 Immunodetection of rhodopsin for assaying POS phagocytosis

A. POS lipid rich disk are composed of rhodopsin, a light sensing molecule. RPE cells do not produce rhodopsin however upon uptake of POS RPE cells become positive for rhodopsin **B.** Over time as RPE first takes up POS they become positive for rhodopsin. **C.**As the RPE cell degrades the POS rhodopsin staining begins to decrease. This assay method shows time dependent degradation of POS phagocytosis by RPE. This conventional method for assaying phagocytosis has been adapted and used in our laboratory. *Yu & Egbejimi et al, Science Signaling, 2018*

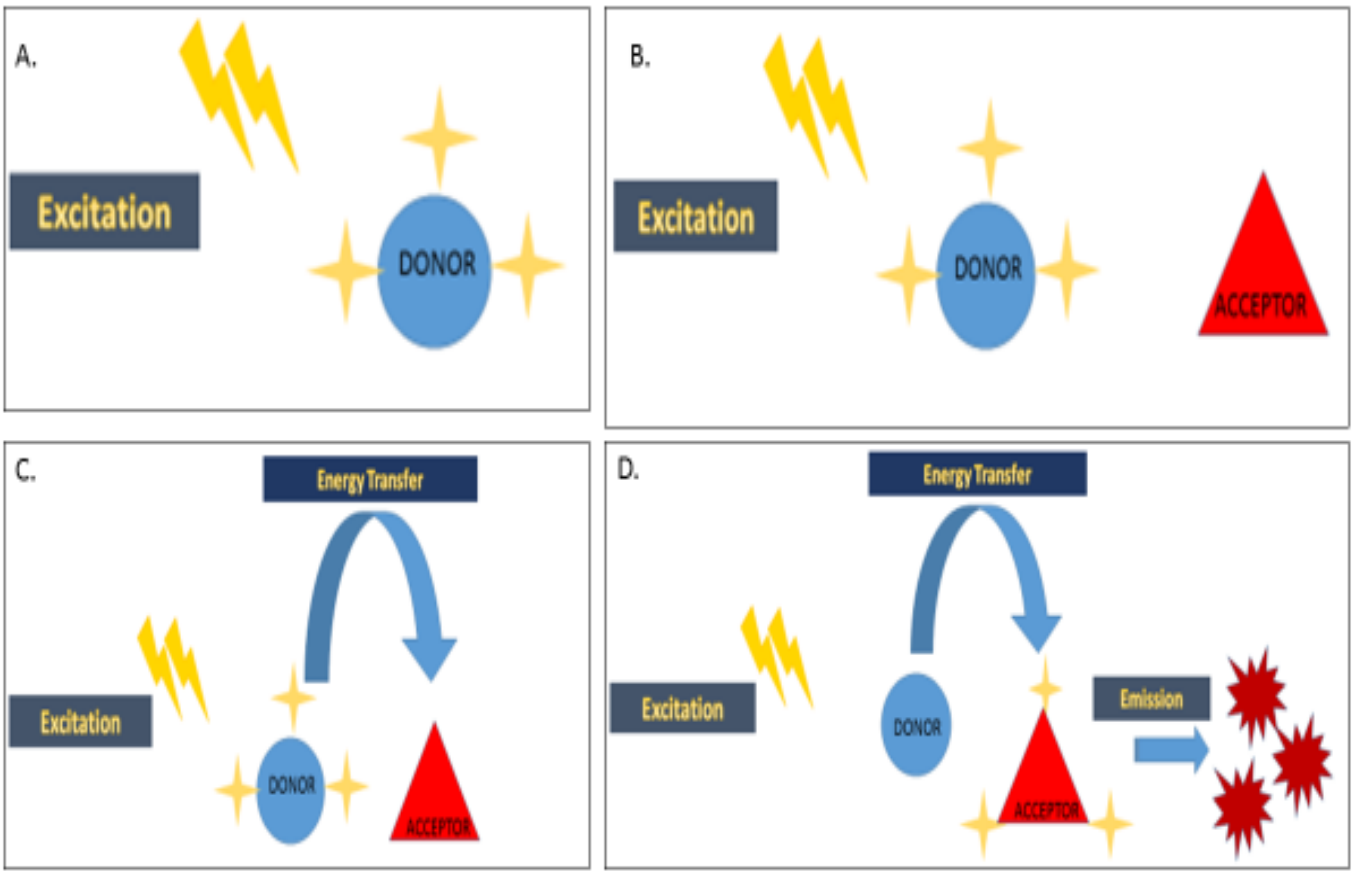

Figure 1. 8 Foster Resonance Energy Transfer

A. At the excitation of the donor molecule it is excited. When the donor molecule comes into proximity with the acceptor **(B),** an energy transfer occurs. **(C)** Fret emission can be observed as the emission of the acceptor and quenching of the donor's emission **(D)**.

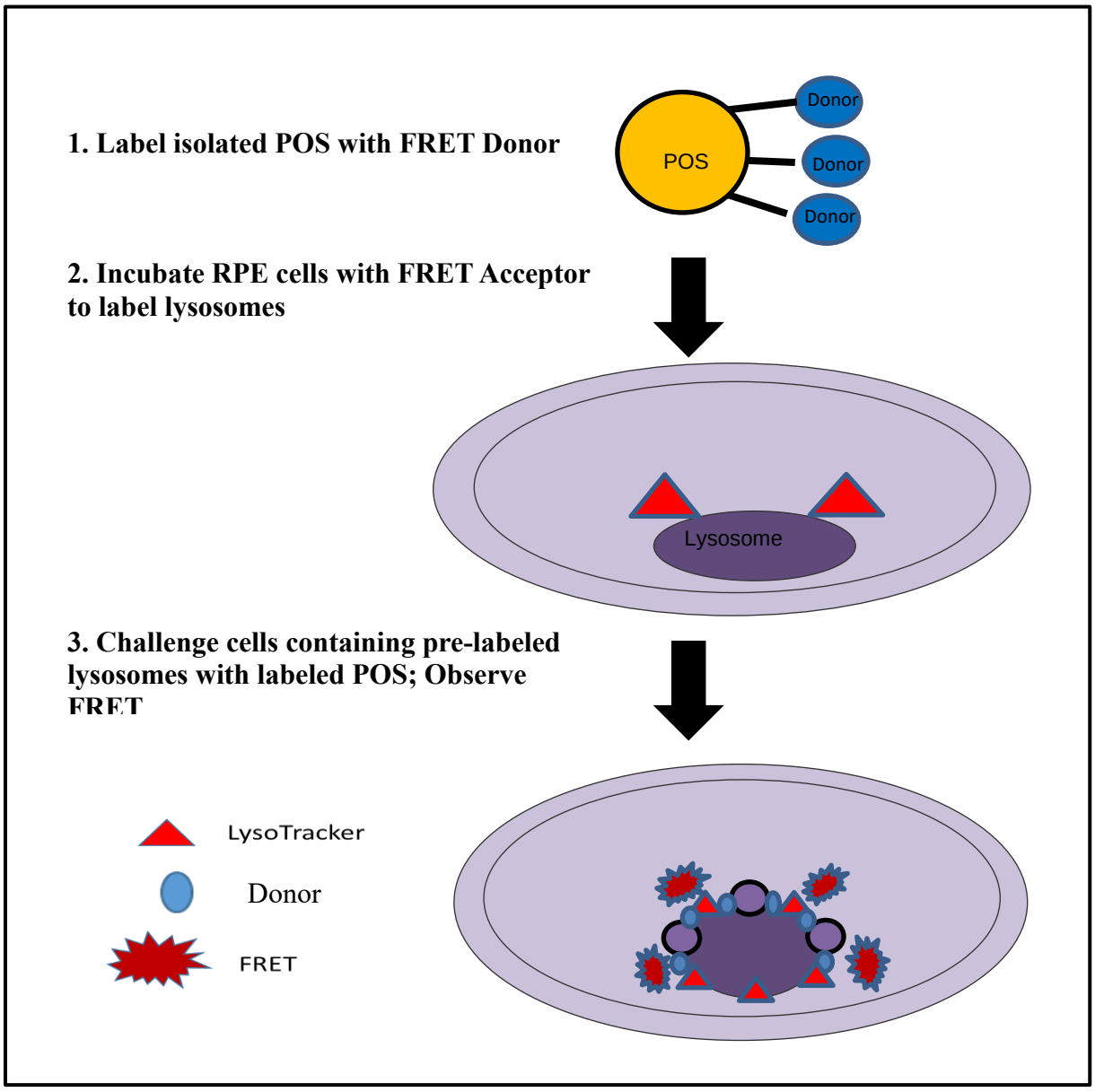

Figure 1.9 FRET Experimental Workflow

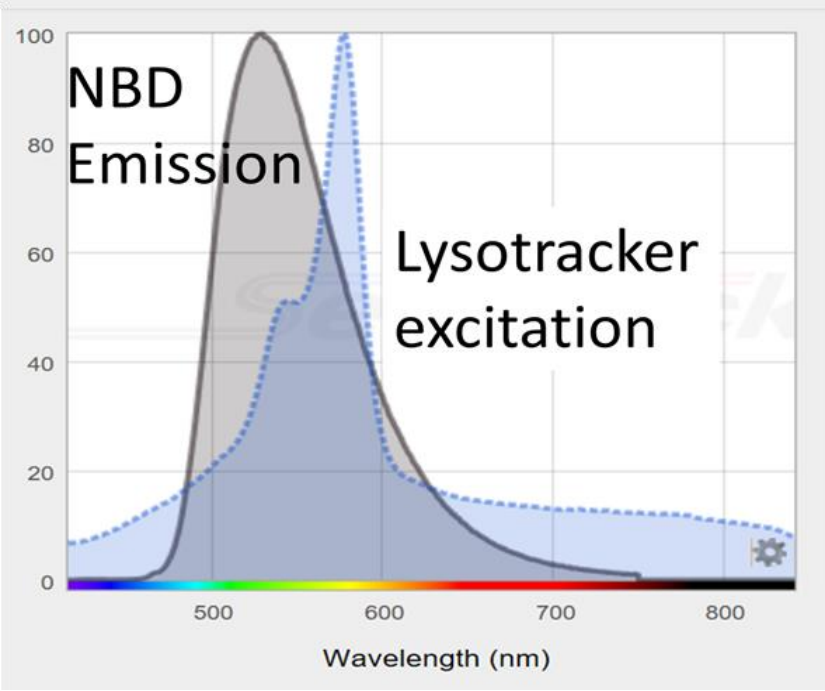

Figure 1.10 NBD and Lysotracker spectral overlap

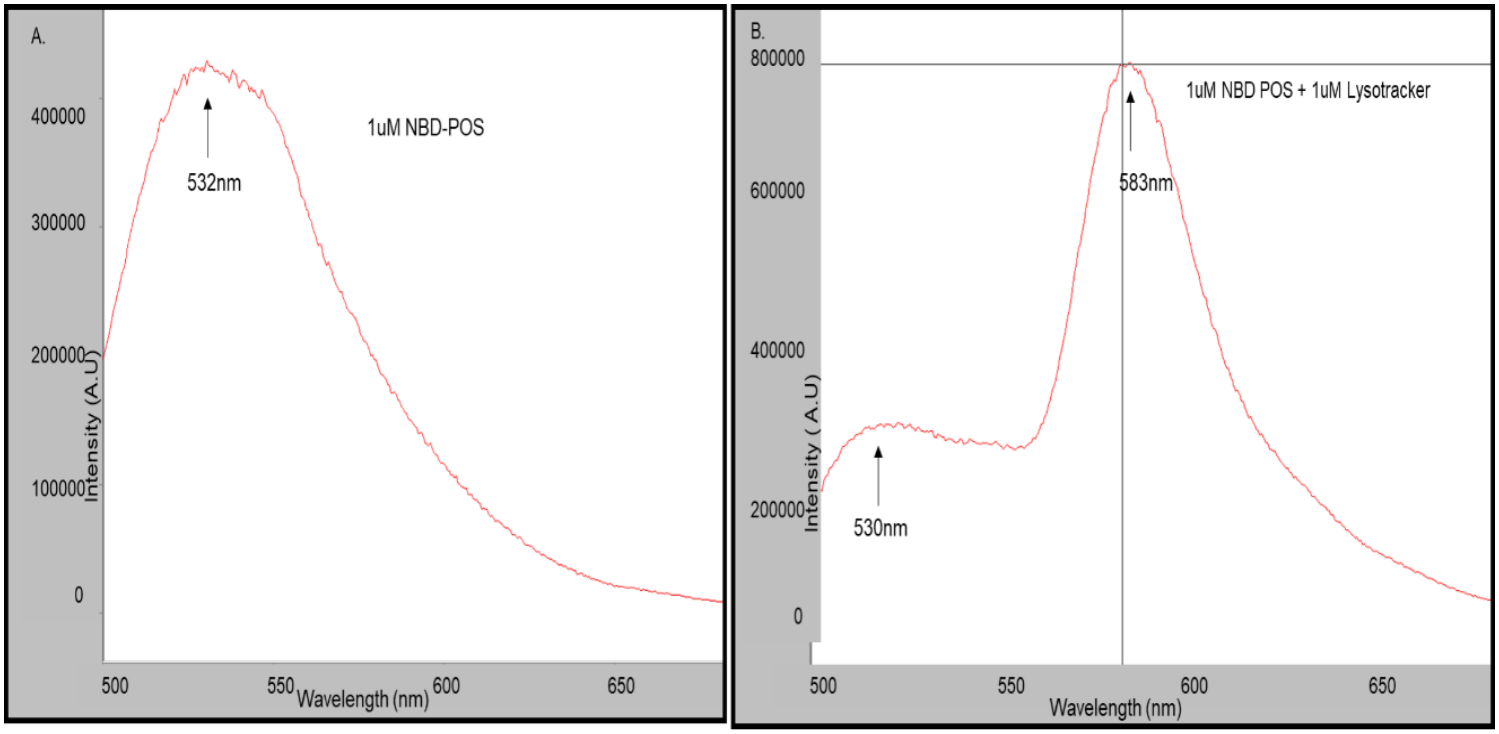

Figure 1.11 NBD- POS and Lysotracker Energy Transfer

A.NBD labelled POS control was excited at 463nm with emission peak collected. Maximum intensity was observed at 532nm, the characteristic emission maximum of NBD. **B.** Equal molar concentration of NBD- POS and lysotracker were mixed in solution and excitated at 463nm with emission curve collected. Maximum intensity observed at 583nm. This depicts the energy transfer from NBD's excitation to lysotracker emission. Experiments were carried out using spectrofluorometer.

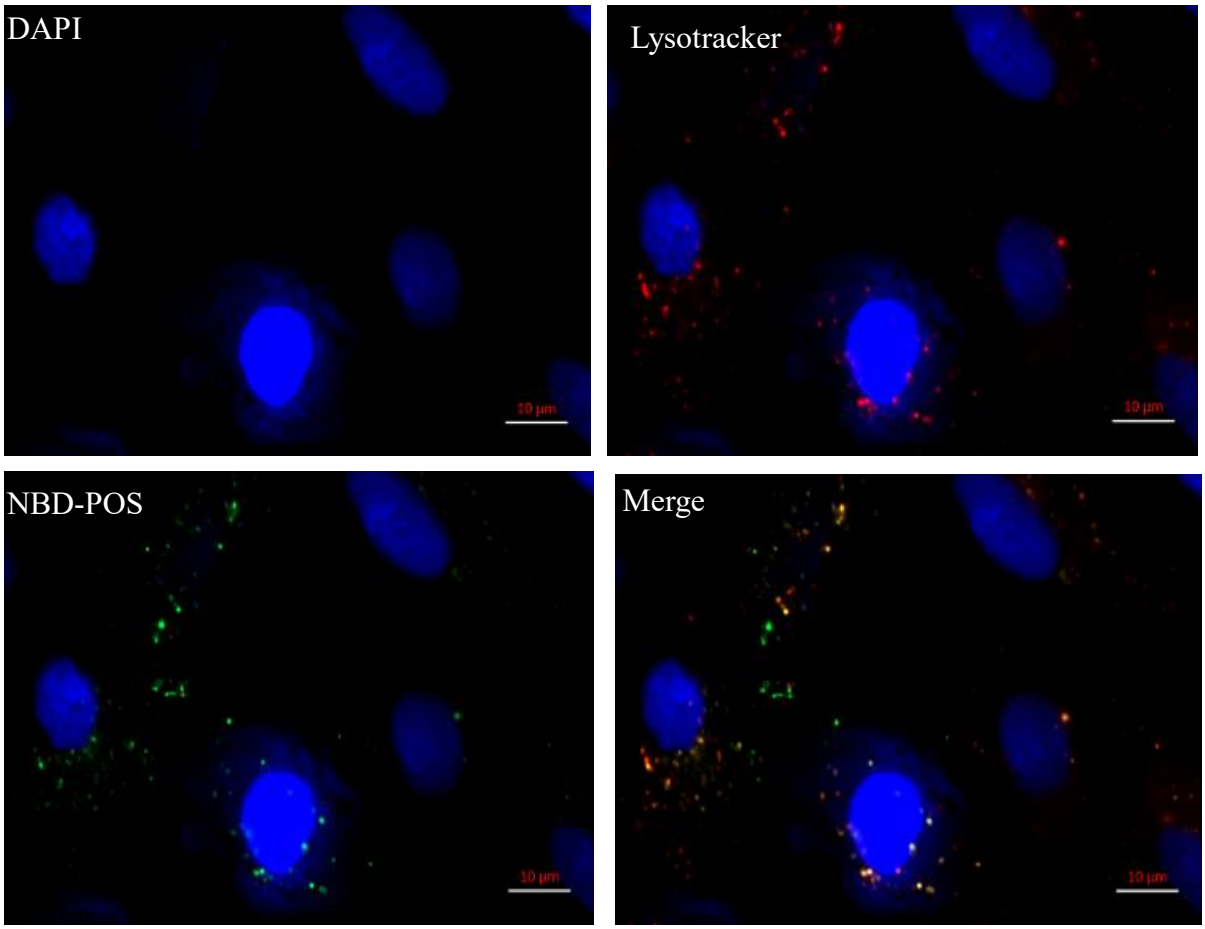

Figure 1. 12 NBD- POS colocalizes with lysotracker labelled lysosome in RPE cells

ARPE- 19 cells were incubated with NBD – POS for 1h and then incubated with lysotracker to label lysosomes. Immunofluorescence detection of NBD- POS shows RPE cells can recognize and uptake NBD labelled POS. Colocalization of NBD-POS to lysotracker labelled lysosomes show uptake NBD-POS can be trafficked in RPE cells. Representative image from multiple experiments. Scale Bar 10um

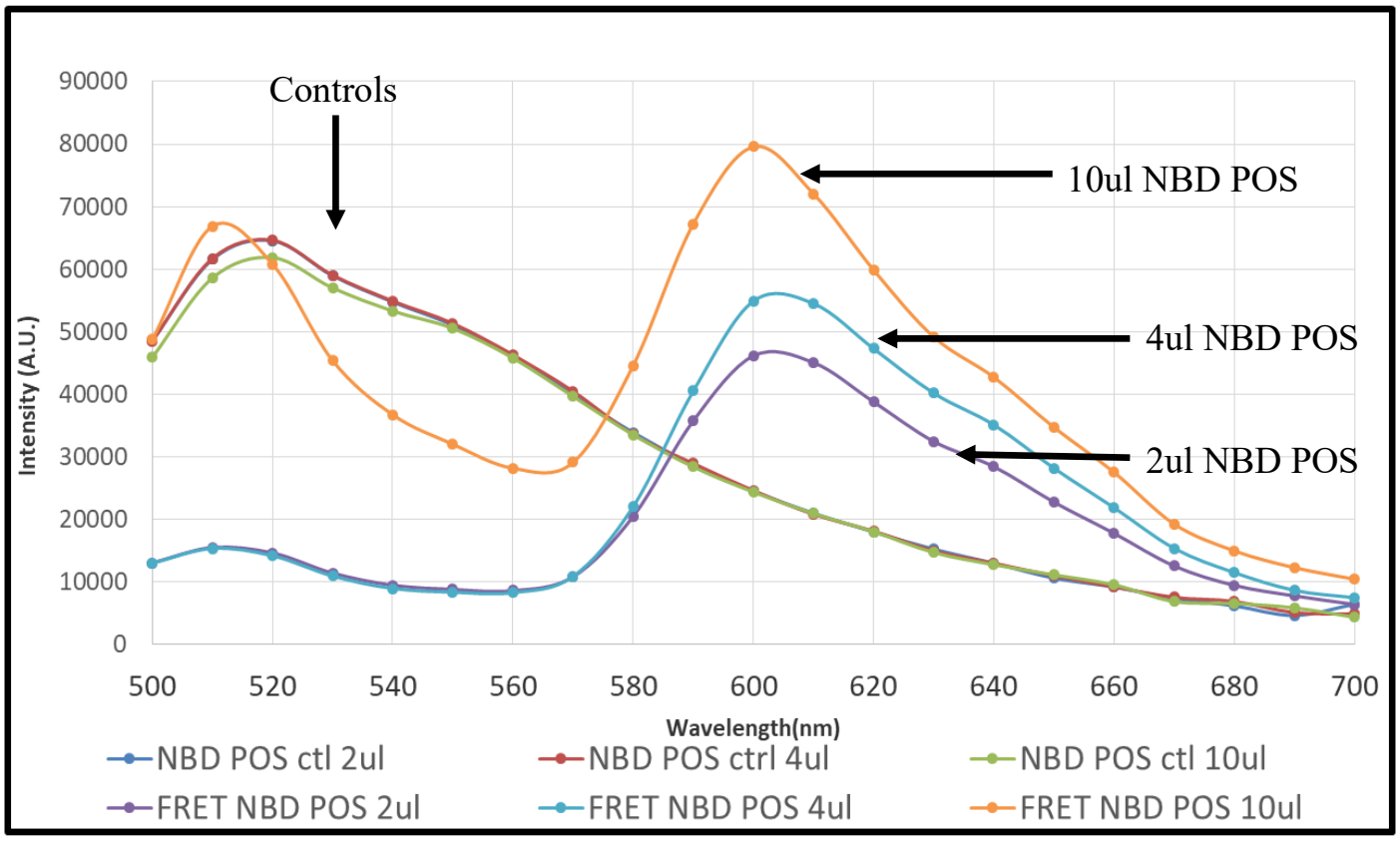

Figure 1.13 NBD - POS caused dose dependent increase in FRET intensity

RPE cells were incubated with NBD-POS at varying concentrations (2ul, 4ul, 10ul). Samples were excited at 463nm, the characteristic excitation of NBD and an emission curve was collected. FRET intensity at 600nm increased in an NBD-POS dose dependent manner. Control samples with NBD-POS had no change in intensity values when excited at the same wavelength. Experiments were carried out using a plate reader.

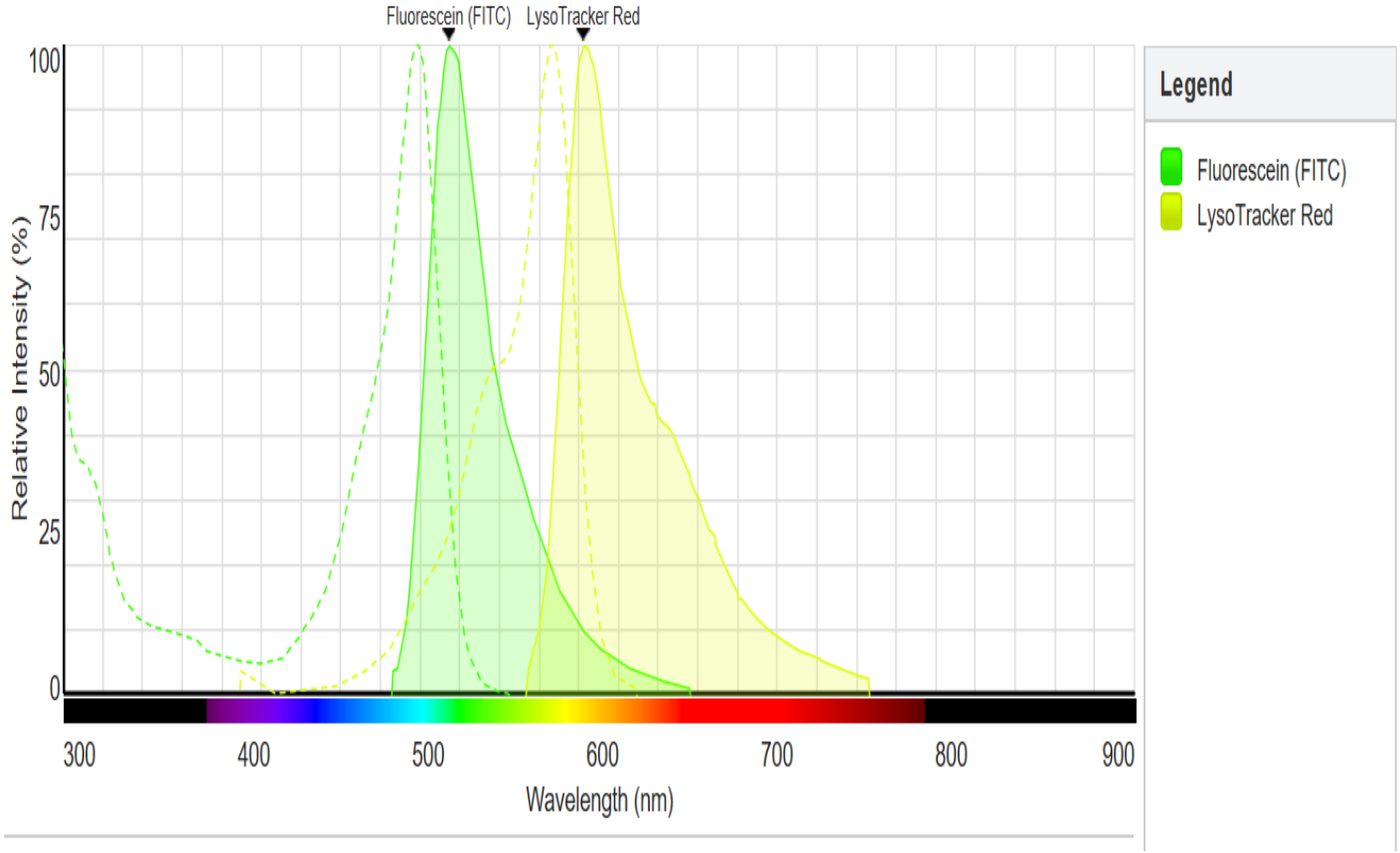

Figure 1. 14 FITC and Lysotracker Spectral Overlap

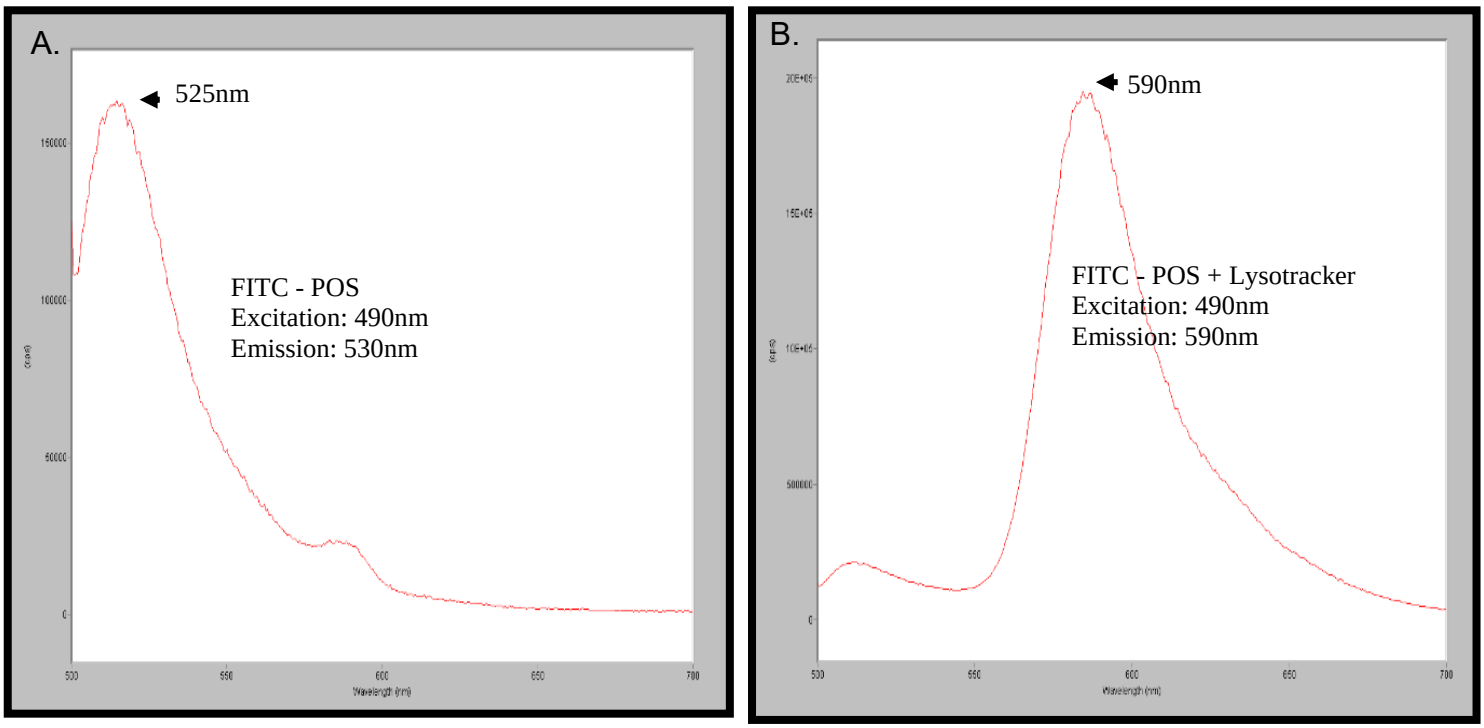

Figure 1.15 FITC- POS and Lysotracker Energy Transfer

A. FITC labelled POS control was excited at 490nm with emission peak collected. Maximum intensity observed at 530nm, the characteristic of emission maximum of FITC. **B.** Equal molar concentration of FITC- POS and lysotracker were mixed in solution and excited at 490nm with emission curve collected. Maximum intensity observed at 590nm. This depicts energy transfer from FITC's excitation to lysotracker emission. Experiments were carried out using spectrofluorometer.

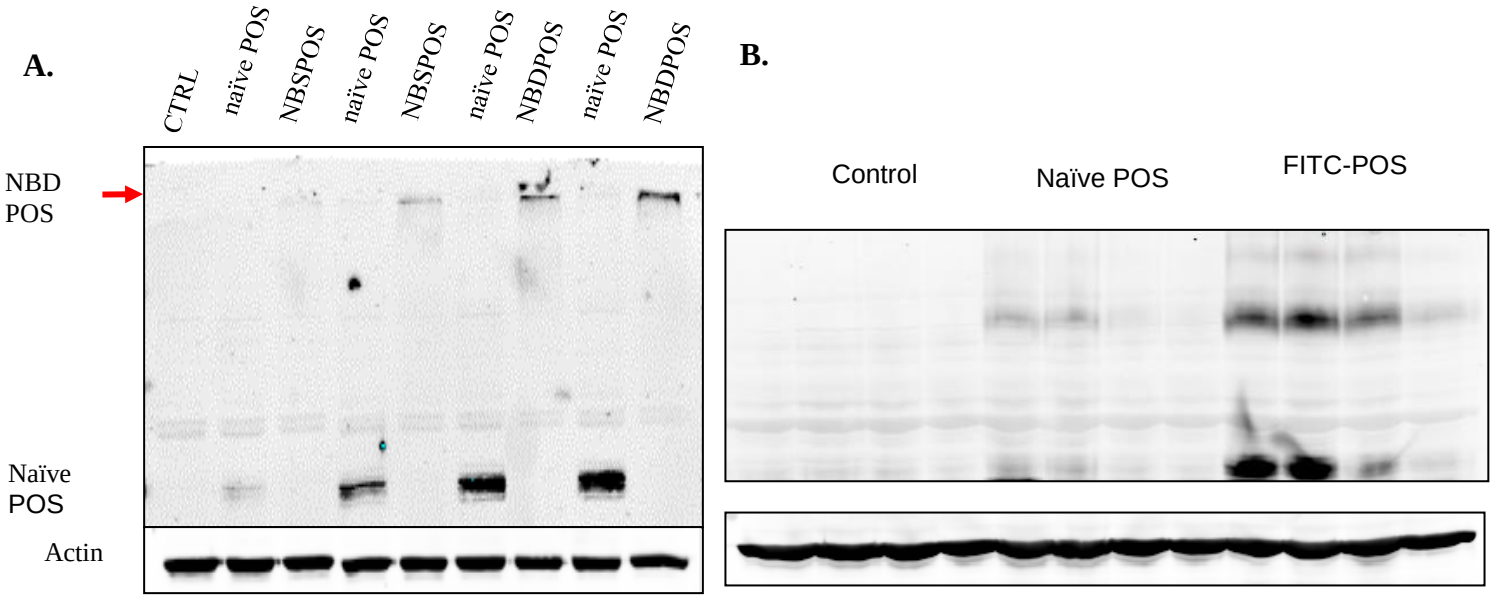

Figure 16 NBD - POS vs FITC POS

A. Immunoblot of 10ul naïve POS and 10ul NBD labelled POS probed with rhodopsin antibody(RET-PA). Naïve POS bands appear at 32kD and NBD POS appear at higher molecular weight (red arrow).**B.** Immunoblot of 10ul naïve POS and 10ul FITC labelled POS probed with rhodopsin antibody.

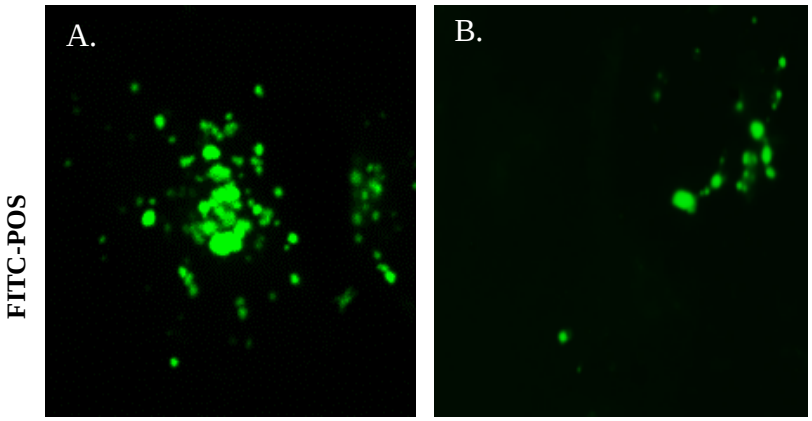

Figure 1.17 Optimization of FITC – POS

A. FITC POS labelling following conventional protocol.**B.** FITC POS labelling following optimization protocol

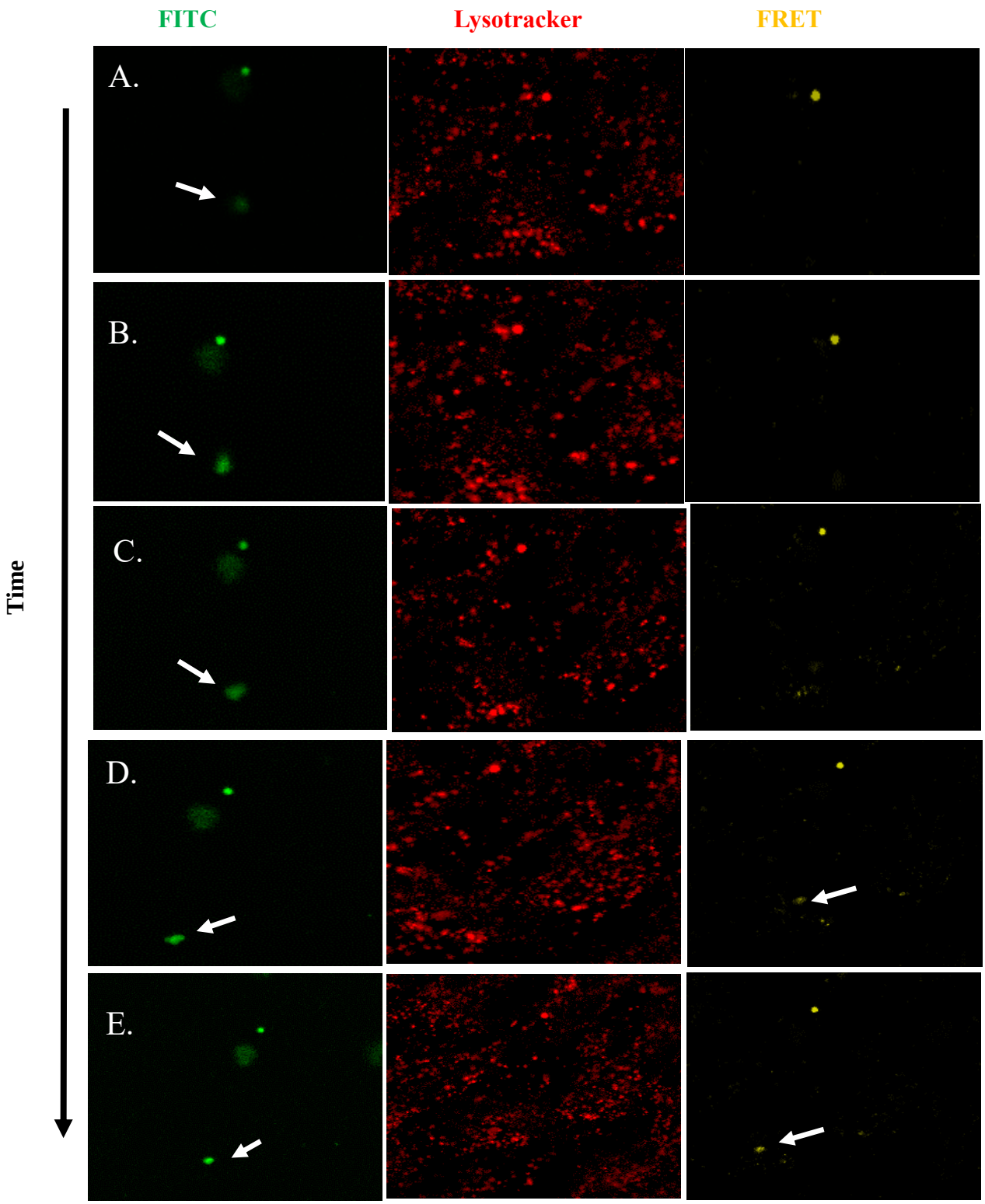

Figure 1.18 Time lapse of FITC- POS movement and fusion with lysosome

FITC-POS movement (white arrow) is observed in A – C. FITC POS causes signal in FRET channel (D & E) as it fuses with lysotracker labelled lysosome

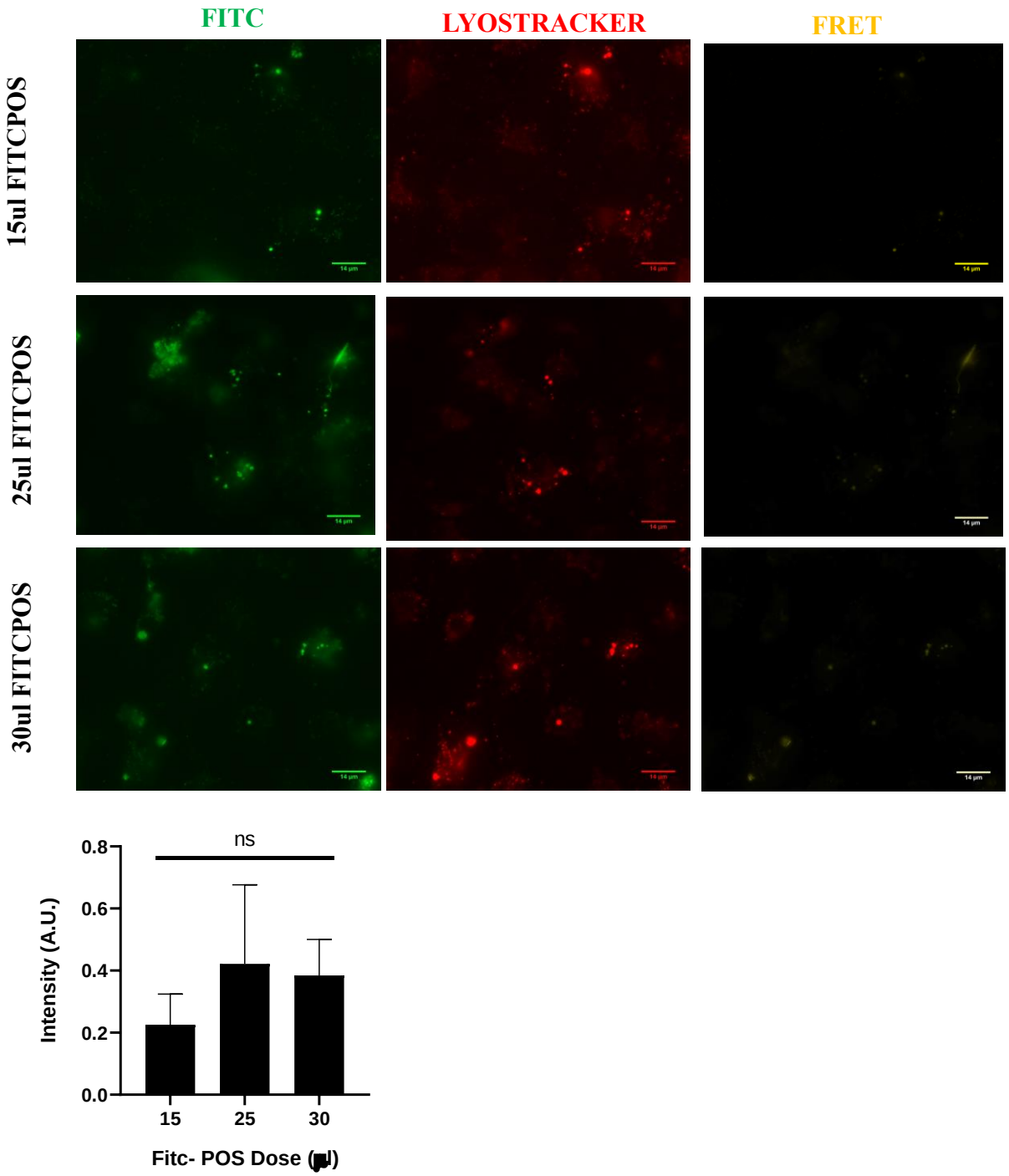

Figure 1.19 FITC - POS caused dose dependent increase in FRET activity

ARPE-19 cells were challenged with increasing doses of FITC- POS (15ul, 25ul and 30ul). FITC- POS causes dose dependent increase in FRET intensity observed in FRET channel. Representative images from single experiment. Bar graph shows mean intensity values with SEM from different areas in different experiments.

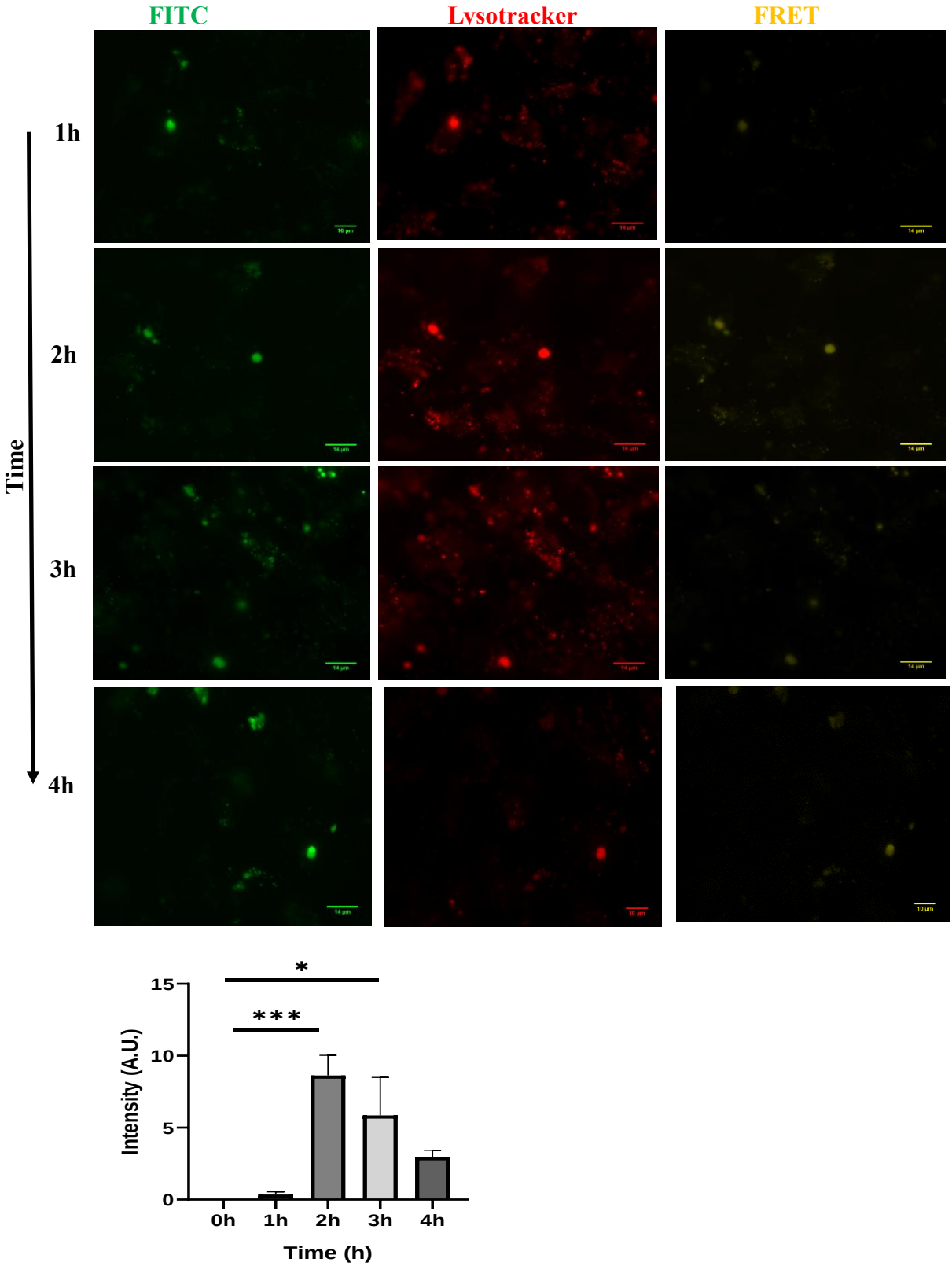

Figure 1.20 FITC - POS time dependent increase in FRET activity

ARPE-19 cells were challenged with FITC- POS at different time points (1h – 4h). A time dependent increase in FRET intensity is observed in the FRET channel. Images were captured at same setting. Representative images from single experiment. Bar graph shows mean intensity values with SEM from different areas in different experiments. N = 3, One – way anova,

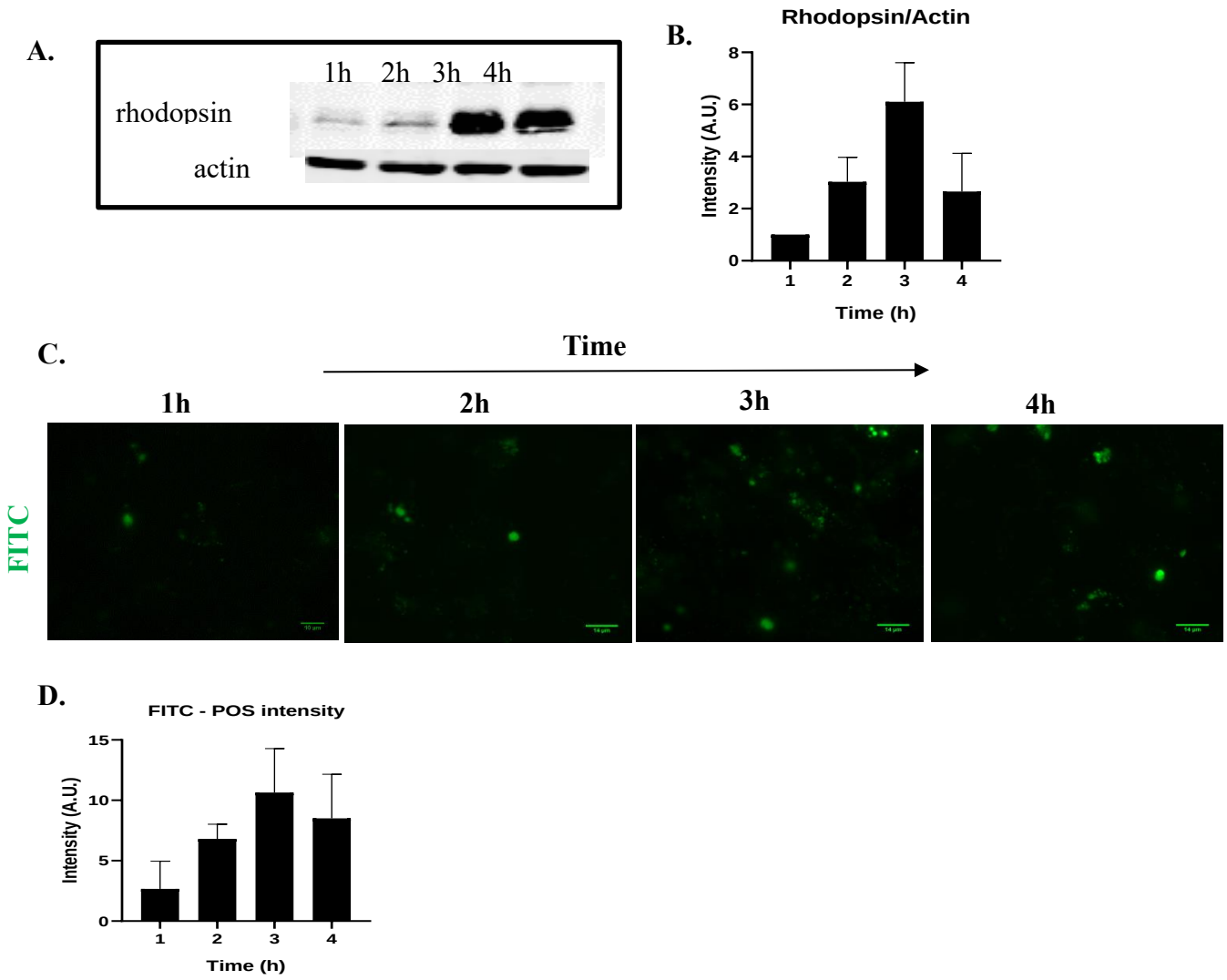

Figure 1.21 FRET assay measures trafficking but not uptake of POS

A. ARPE-19 cells challenged with FITC- POS at different time points, cells were harvested to measure uptake of POS via rhodopsin immunoblot (rhodopsin antibody RET-P1). **B.** Quantification of intensity values rhodopsin intensity values normalized to actin with SEM from different experiments. (n = 3). **C.** FITC-POS intensity at different time points in ARPE-19 cells. Representative images from single experiment **D.** Mean intensity values from different experiments and different areas.

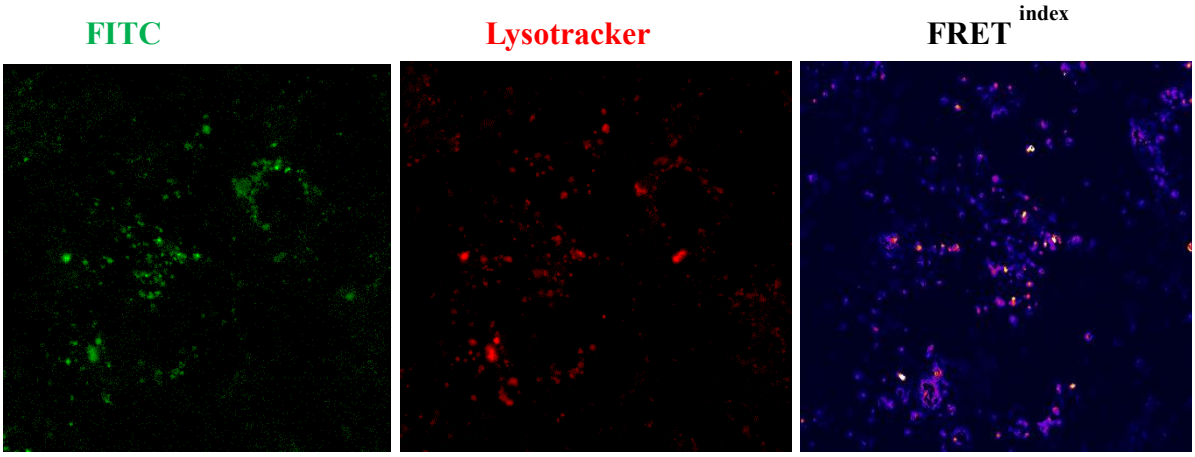

Figure 1.22 FRET ^{index}

Using Image J Plug-In, FRET & Co- localization analyzer, FRET index was obtained

Table 1 FRET Validation

Distinct Step	Factor for Validation	FRET signal
Trafficking to lysosome	Atg5 knockdown	⬇
Degradation	Chloroquine treatment	⬆

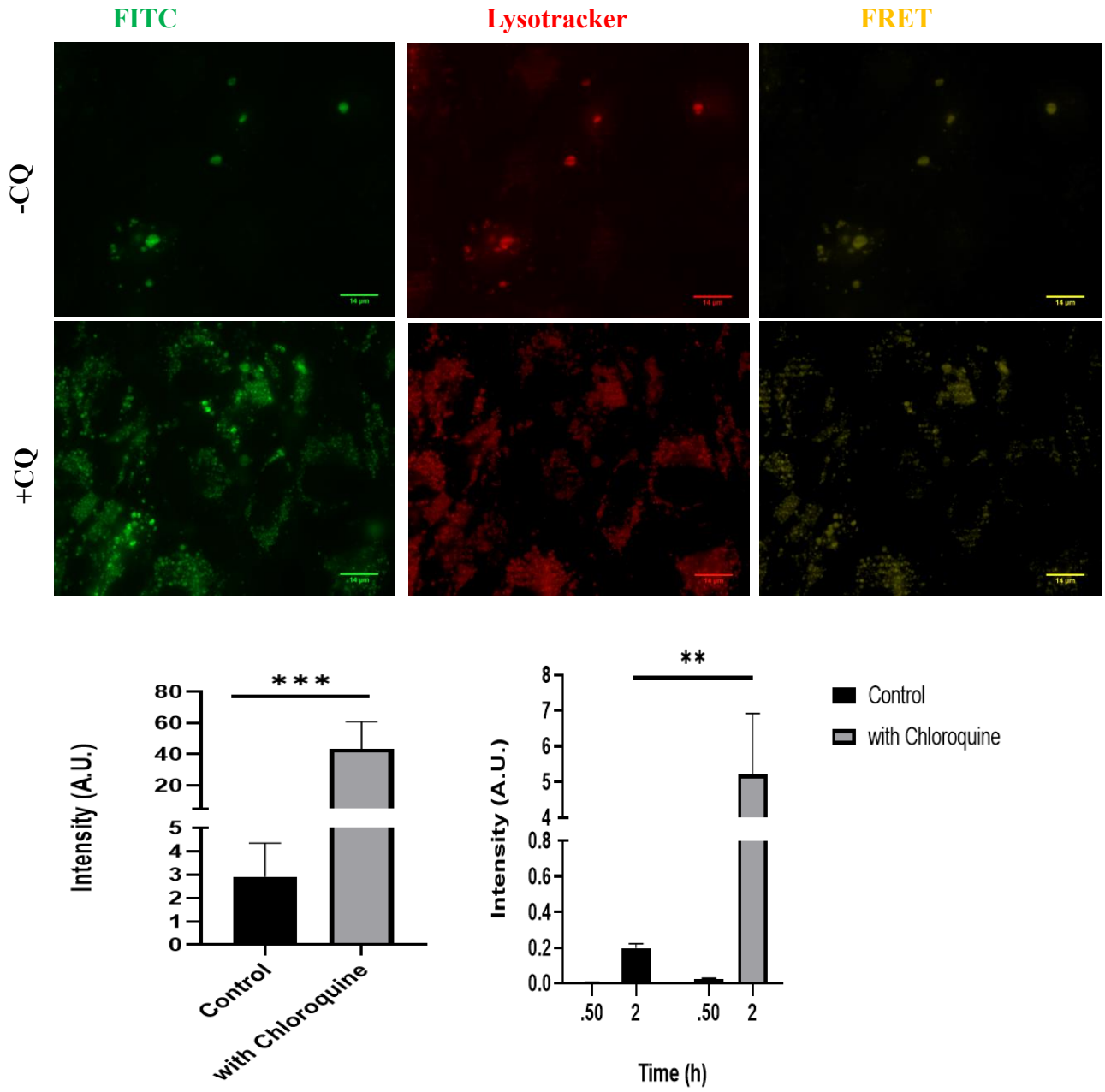

Figure 1.23 Chloroquine treatment enhanced FRET signal

A. ARPE-19 cells were treated with Chloroquine before being challenged with FITC- POS and incubated with lysotracker to label lysosomes. Representative image from single experiment. **B.** Mean intensity values with SEM for FRET intensity from different experiments and different areas. N= 3, One – way ANOVA,**C.** Mean intensity values with SEM for FRET intensity derived from time course experiments. N=3, Two – way ANOVA,

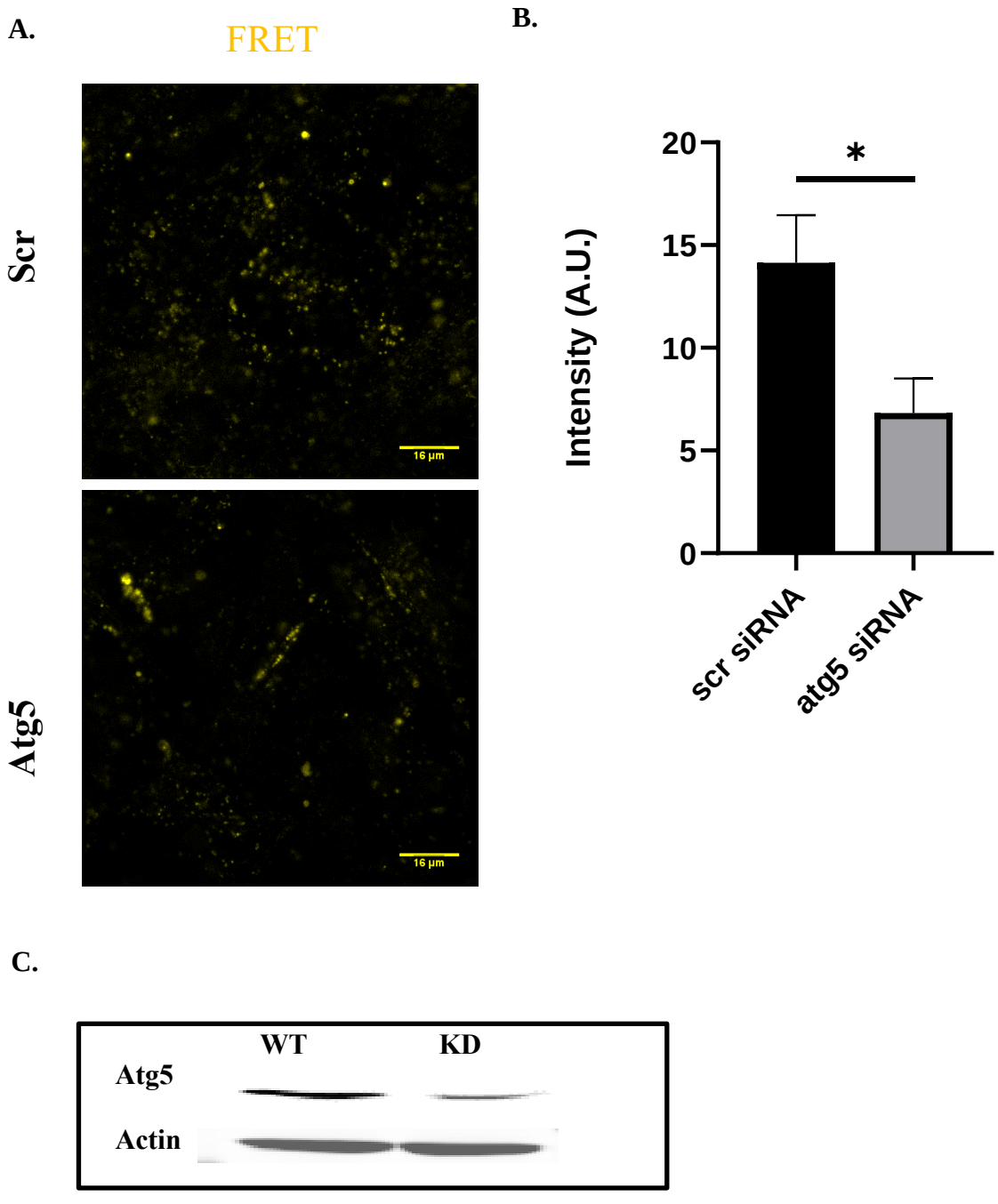

Figure 1.24 Atg5 knockdown caused reduced FRET signal

A. Atg5 knockdown ARPE-19 cells were challenged with FITC- POS . Representative image from single experiment. **B.** Mean intensity values from FRET channel with SEM from different experiments. N = 3, One- way ANOVA, **C.** Western blot of Atg5 knockdown

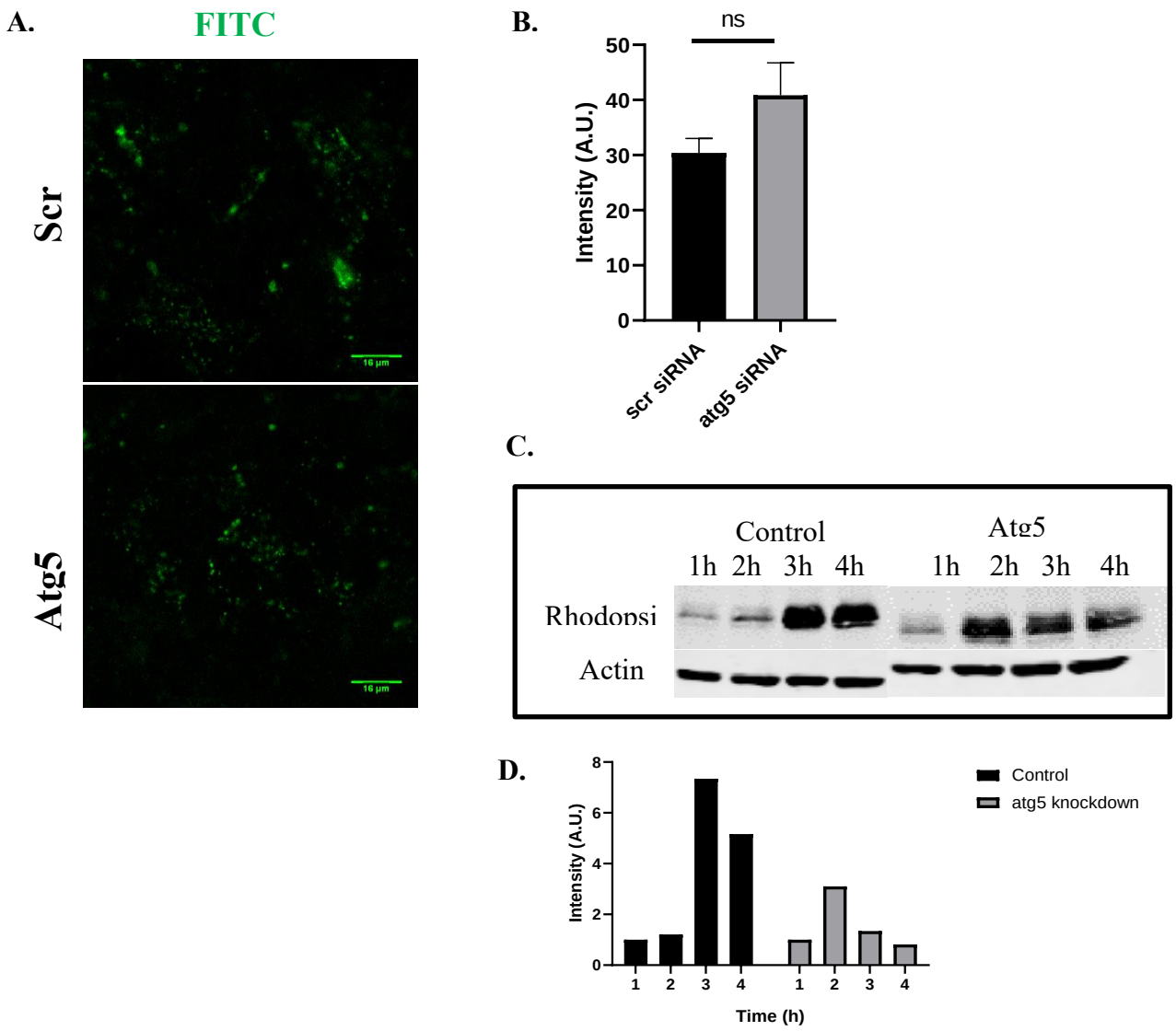

Figure 1.25 Knockdown of Atg5 does not affect POS uptake

A. FITC -POS intensity in Atg5 knockdown cells. Representative image of single experiment. **B.** Mean intensity values of FITC- POS channel with SEM from different experiments. N = 3, One- way ANOVA **C.** ARPE-19 cells challenged with FITC- POS at different time points, cells were harvested to measure uptake of POS via rhodopsin immunoblot (rhodopsin antibody RET-P1). **D.** Quantification of intensity rhodopsin intensity values normalized to actin with SEM from different experiments.

Table 2 Primary Antibodies

Protein	**Catalog Number**	**Company**
Atg5		
β actin	*ab8229*	*Abcam*
Rhodopsin	*ab3267*	*Abcam*
VPS11	*ab170869*	*Abcam*
VPS18	*sc-100890*	*Santa Cruz Biotechnology Inc.*

Chapter 3: Identification of Novel regulators of Photoreceptor Outer Segment trafficking in RPE

Introduction

Phagocytosis of shed photoreceptor outer segment (POS) is an essential process to prevent accumulation of extracellular debris in the retina. Clearance of ingested POS ensures degradation of certain components and recycling of useful material. The RPE's unique features makes the clearance pathway a unique phagocytic process. Undigested POS can lead to build of lipids and cause oxidative stress within the RPE. Other age associated changes within the RPE can contribute to inefficient clearance, resulting in accumulation of extracellular and intracellular debris. Elucidating the molecular machinery responsible for efficient clearance of ingested POS has been a major goal in the field of RPE biology. Identifying the regulators of this phagocytic process would not only allow us to properly characterize phagocytosis in RPE but would potentially lead us to new targets for treatment of RPE loss and retinal degenerative diseases. Much of our understanding of the phagocytic process is borrowed from traditional phagocytes. Phagocytosis of POS in RPE shares similarity with traditional phagocytosis in other cells. However, because RPE is a highly specialized group of cells there remains distinctions between phagocytosis in RPE and traditional phagocytosis.

The trafficking step of the POS phagocytosis pathway is a crucial step which ensures phagosome fusion with lysosome. Without phagolysosome formation, ingested POS will be improperly cleared and accumulate intracellularly. Thus, it is important to uncover the mechanism of phagosome fusion with lysosome in the RPE. Our goal is to identify novel regulators of the trafficking step of POS phagocytosis in RPE. Specifically,

we performed a literature search to identify candidate genes which may play a role in trafficking of POS in RPE. Our candidate genes were selected based on their role in the endocytic pathway. The candidate genes selected were then assessed primarily with the novel trafficking assay we developed.

Background

ENDOCYTIC PATHWAY AND ENDOCYTIC TRAFFICKING

The endocytic pathway in eukaryotic cells in responsible for cellular internalization of macromolecules and proteins which lay at the surface (54- 56). Endosomes play a critical role in regulating biological processes such as nutrient uptake, immunity, signaling, membrane turnover and development (54,55). Communication between cells and the environment is achieved largely through endocytosis. Through endocytosis the cell regulates the levels of various essential surface proteins and transporters important in human health and diseases. For example, glucose transporters maintain serum glucose levels and proton pumps help control stomach acidification (54). Endocytosis also regulates signaling of various surface receptors such as G- protein coupled receptors and receptor tyrosine kinases via uptake of integrin receptors and adhesion molecules (54). Materials that has been endocytosed move through various pathways to be delivered to an early endosome (EE) (54). EE is also thought of as the sorting endosomes since molecules are segregated and sorted at this point. Two major and distinct pathways first identified for endocytosis are clathrin mediated endocytosis (CME) and caveolae uptake (54). Other pathways were later discovered such as cholesterol sensitive clathrin and caveolae independent pathways, clathrin independent carriers (CLIC) and glycophosphatidylinositol-anchored protein-enriched endosomal compartments (GEEC)(54).

Clathrin mediated endocytosis is the most studied and most well understood mechanism for uptake into cells. CME occurs through clathrin – coated pits (CCPs) and clathrin coated vesicles (CCVs). Clathrin is a scaffold protein consisting of a light and heavy chain. Clathrin assembles to form a coat around a mature vesicle which forms from the invaginated membrane (54). The coat dissociates rapidly once the vesicle is detached from the membrane. CCP is assembled through recruitment and accumulation of

phosphatidylinositol- 4,5, - bisphosphate (PIP2) and AP2 to the plasma membrane (54). The growth and stability of the complex requires BAR (Bin – Amphiphysin- Rvs) , actin and dynamin. Once the vesicle is dissociated it travels to fuse to a targeted endosome.

CavME or caveolae – mediated endocytosis is the second most studied uptake mechanism. This mechanism of endocytic uptake is directed by caveolae, a flask shaped plasma membrane invagination approximately 50 – 100nm in diameter (54). These caveolae are found to be present on many eukaryotic plasma membranes. The main family of protein found within caveolae are caveolin with caveolin-1 being the most common (54). Caveolin-1 and other members of caveolin proteins are embedded within the inner leaflet of the plasma membrane bilayer (54). The caveolin proteins function as a scaffold binding with other proteins known as cavins as well as cholesterol and other important signaling molecules (54). CaveME is initiated by ligand binding to cargo receptors within the caveolae. Budding of the caveolae is regulated by kinases and phosphatase and dynamin is also known to be required for the dissociating of caveolae vesicles from the plasma membrane (54).

These two major pathways for endocytic uptake have been well studied and characterized, however, recently it has been discovered that there are other pathways for endocytic uptake which different mechanistically from CME and CavME. These pathways do not require clathrin or caveolin, as they were first discovered by their resistance to inhibitors which block CME and CavME (54). These pathways have not been well characterized and therefore are not completely understood (54). It has also become clear that CME and the clathrin independent endocytic (CIE) pathways are differentially regulated and therefore functionally different and independent of each other (54). In other words, cells may use both CME and CIE pathways for various cargos for different reasons.

After material has been internalized through these various pathways it may be directed either to the lysosomes for degradation, recycling back to the plasma membrane or sent across the cell through transcytosis (54). The early endosome goes through various

stages of maturation into a mature and late endosome. These stages involve changes in ph, and changes in production of phosphatidylinositol lipids, and activation of Rab family GTPases (54,55). The fate of the internalized cargo is directed by the endosomal network. The network allows trafficking of material through the cell via transfer between various membrane bound compartments. During the intricate steps that follow internalized cargoes are sorted and then distributed to their final destinations. Following uptake and internalization, the endocytic vesicles which are formed undergo homotypic fusion to form early and sorting endosomes (54,55). Cargo destined for recycling at the plasma membrane are sorted through the recycling endosome. Other internalized cargo may be sent to the trans-Golgi network (TGN) via retrograde trafficking (54,55). Cargo destined for the lysosomes for degradation maybe trafficked through the late endosome or multivesicular body (MVB) (54). MVB formation is directed by the ESCRT (endosomal sorting complex required for transport) complex which recognizes ubiquitin tags on cargo and ensure proper sorting (57). There have been four different ESCRT complexes identified directing a specific step of MVB formation (57). Each endocytic compartment has a unique signature of PIPs and Rab family GTPases which changes as cargo is transferred and exchanged between endosomes. This process is known as endosome maturation. Early endosomes mature to late endosomes as their lumens becomes more acidic. The v-type vacuolar H + ATPase (V- ATPase) within the membrane bilayer pumps H+ ions into the vacuole lumen, decreasing the pH (54-55,58). PIPs and Rab family GTPases are also important in the endosomal maturation process. Rabs are important in providing structural and functional identity to endosomes and help to recruit effector proteins to the surfaces of membrane compartment (54,55). Rab proteins can be active or inaction depending on the factor bound. GDP bound Rab is in an inactive state while the GTP bound counterpart is in an active state (59). This conversion from inactive to active is carried out by a specific guanine nucleotide exchange factor (GEF) (59). GTP bound activated Rab protein interaction with

various tethering factors is a crucial event in the endosomal fusion and maturation process (59).

Endocytic sorting is also an important process in the endomembrane system. Sorting of internalized cargo is directed mainly by endosomal acidification and ligand dissociation as well as the endosome geometry (54).

The homotypic and heterotypic fusion of the EE is controlled by SNAREs (55). EE SNAREs include syntaxin 13, syntaxin 6, and Vamp4 (55). Fusion in EE also requires regulatory factors such as Rab5 GTPase. Two larger sets of tethering complexes which are involved in endosome maturation are the HOPS (late endosomal) and COVERT complex (55,57). These complexes contain the same distinct subunits. The COVERT and HOPS complex have been shown to be interchangeable in response to Rab5 – Rab7 transition (55,57). The CORVET and HOPS complex tether membranes via their interactions with Rab GTPases and activation of SNARE assembly (57). The complexes have also been shown to attach endosomes to the cytoskeleton.

Because of the universal and critical role of endocytosis, it is no surprise that inhibition of the endocytic pathway can be unfavorable to the cell. Loss of function of principal components in CME such as dynamin and AP2 cause embryonic lethality (54). Although there are no known severe mutations in some of these critical players in CME in human disease, aberrations of CME have been described in various human diseases like cancer and neurodegeneration (54). Some anomalies in CavME have also been reported in some human diseases. It is no wonder also that CIE pathways are also thought to be implicated in human diseases. Our understanding of the CIE pathways remains a limiting step in understanding the physiological important of the pathway in some human disease. Alterations in lipid phosphates, kinases and Rab family proteins as well as other components of endocytic trafficking and sorting have also been identified in various human diseases (54). The aging process can also alter normal endocytic function in cells (64). Defects in endocytic pathway have also been linked to aberrant storage of undigested

material in lysosomal compartments resulting in inflammation and other apoptotic signaling (65). Lysosomal storage diseases (LSD) are an example of a traffic jams within the endocytic pathway where mutations within the cell impair hydrolases and lysosomal compartments. LSD lysosomal dysfunction causes various organ damage, developmental abnormalities and central nervous system degeneration as well as premature mortality (65).

VACUOLAR PROTEIN SORTING SUBUNIT 11 (VPS11)

Homotypic fusion and protein sorting (HOPS) and class C core vacuole/endosome tethering (COVERT) are conserved protein complexes involved in endosomal fusion (44-46). HOPS and COVERT were first identified in yeast but are now known to be important for vacuolar/lysosomal fusion in mammals. HOPS and COVERT consists of the same vacuolar protein sorting subunits (VPS) 11, 16, 18, 33,39, and 41. COVERT is associated with early endosome fusion via binding of Rab5 GTPase (46, Figure 2.1). HOPS is associated with late endosome via replacement of Rab5 with Rab7. The structure of HOPS and COVERT complex is depicted in Figure 2.1. Fusion to the lysosome has been shown to be impaired in knockdown of components of the HOPS and COVERT complex (44).

There are more than 40 vacuolar protein sorting (vps) genes. VPS11 is part of the COVERT and HOPS complex core subunit. Loss of VPS11 has been shown to cause retinal degeneration phenotypes in oculocutaneous albinism vertebrate model (44-45). VPS11 and other proteins involved in trafficking have been studied in yeast. VPS11 physically interacts with VPS18, VPS16 and VPS33 (45, Figure 2.1). In yeast, loss of VPS11 resulted in formation of abnormal vesicles and an accumulation of the intermediate transport vesicles (44). Yeast vacuole are comparable to vertebrate lysosomes and melanosome, therefore VPS11 has been hypothesized to play a role in endosome to lysosome trafficking. Moreover, retinal degenerative phenotypes observed in mutant VPS11 zebrafish support the hypotheses that VPS11 is important for vesicle transport (44). For example, vps11 mutant zebrafish showed progressive loss of RPE pigmentation at different developmental stages. Additionally, rhodopsin was mis – localized in mutants corresponding to truncated photoreceptor outer segment compared to wild type. Lastly, electron microscopy showed accumulation of vacuoles containing rod outer segments in mutant. Thus, vps11 loss or

mutation likely causes a toxic accumulation of proteins not properly trafficked within the RPE.

VACUOLAR PROTEIN SORTING SUBUNIT 18 (VPS18)

Vps18, another major component of the HOPS and COVERT complex, has also been shown to regulate autophagosome and late endosome formation (43). Zebrafish mutant vps18 shows similar RPE dysfunction as observed in vps11 (47). RPE cells in the mutant zebrafish accumulated immature melanosomes (47). The vps18 mutant model exhibited vesicle trafficking defects and suggest that the COVERT and HOPS complex play a crucial role in trafficking in RPE. VPS18 conditional knock out in mice brain was reported to accumulate autophagosome, suggesting blockage of autophagosome clearance or lysosomal fusion (45). Endocytic pathway markers were also upregulated in the vps18 conditional knockout. These data further prove that the HOPS and COVERT complex are crucial for endocytic pathway function and thus could be implicated in trafficking in RPE.

The arrangement of protein subunits within the HOPS and COVERT complex has been studied. It has been shown that both complexes require all six subunits for activity (48). Loss of vps11 subunit caused a complete loss of the HOPS complex (48). Therefore, ablation of vps11 or vps18 subunit is suggested to lead to loss of the complexes.

It has recently been shown that both vps11 and vps18 contain a c – terminal RING domain which is characteristic of E3 ubiquitin ligases (62), suggesting that vps11 and ps18 interaction is involved in some signal transduction events (62). It has also been shown through structural studies that the vps18 interacts with vps11 at the N – terminal domain and can also bind to lipids (63). These are important considerations for POS phagocytosis in RPE and can offer us some molecular insight to the role of these vps subunits in the unique phagocytic pathway. The same study also reported that inhibition of the vps18 subunit alone did not impede the assembly of the HOPS complex however did affect

function and stability (63). This finding further supports our hypothesis and other reported findings that function of the HOPS and COVERT complex is dependent on a physical interaction between vps11 and vps18.

Results

To examine the role of components of the HOPS and COVERT complex in POS trafficking in RPE we used siRNA knockdown of the key subunits. To ensure knockdown of the complexes with performed combination siRNA knockdown of both vps11 and vps18 in RPE cells (Figure 2.2). Using siRNA knockdown, we achieved about 75% knockdown of vps11 and vps18 in our combination knockdown cells. (Figure 2.2 E). Immunofluorescence staining of vps11 and vps18 in vps11/vps18 combination knockdown cells shows significantly reduced intensity (Figure 2.2 A -D). After achieving knockdown of candidate genes, we examined the role of both genes in POS trafficking in RPE using out established FRET assay.

Combination vps11/vps18 knockdown cells were challenged with FITC- POS for 2 hours. After incubation cells were washed and incubated with lysotracker to label lysosomes. Cells were then live imaged as before. FRET intensity was calculated using Image J with previously established protocol. Figure 2.3 shows FRET intensity values of knockdown cells compared to scrambled. FRET intensity significantly decreased in vps11 and vps18 combination knockdown cells. The reduction in FRET intensity at the 2-hour time point did not correlate to uptake of POS. POS uptake in vps11/vps18 knockdown cells at 2 hours was not changed compared to scrambled control cells. Thus, vps11/18 knockdown in RPE did not affect uptake of POS.

Discussion

Vps11 and vps18 are essential subunits of the HOPS and COVERT complex. The HOPS and COVERT complex are key effectors in endocytic trafficking. COVERT complex is important for early endosome and is a Rab5 associating complex. HOPS is a late endosome and endo-lysosomal fusion and is a Rab 7 associating complex. Both complexes contain the same essential six subunits. At the core of the complex is vps11 and vps18 , which have been shown to be required for activity of both COVERT and HOPS complex. In studies with mutant vps11 and vps18 it has been shown that both subunits are essential for proper development and function of RPE. WE therefore hypothesized that the endocytic trafficking subunits are crucial in POS trafficking in RPE. To test our hypothesis, we performed combination siRNA knockdown of both vps11 and vps18 to impede formation of the HOPS and COVERT complex. Our results suggest that disruption of these complex leads directly to reduced POS trafficking in RPE. However, uptake of POS is not affected. Knockdown of vps11 and vps18 inhibits phagosome fusion with lysosomes in RPE. Our observed results is similar to the observed accumulation of melanosomes in vps18 (45) mutant and accumulated vacuoles in vps11 mutant in zebrafish (44).

Disruption of endocytic trafficking complex therefore leads to accumulation of ingested but not cleared POS in RPE. These findings help us uncover the role of endocytic proteins in the unique phagocytic pathway in RPE. Future mechanistic studies can help to further describe the role of vps11 and vps18 in POS trafficking in RPE.

Considerations for future projects would be the function of tethering complexes within the RPE and the role these complexes play specifically in POS phagocytosis. Our studies showed that loss of vps11 and vps18 subunits did not alter the RPE's ability to internalize POS fragments. These findings are surprising since COVERT and HOPS complex been previously shown to be involved in EE fusion. The findings thus provide new insight into the specific roles of the complexes in the endocytic pathway. Furthermore,

future studies can aim to uncover the mechanism by which disruption of the formation of the COVERT and HOPS complex result in accumulation of undigested POS fragments within the RPE.

Our study also provides a notable link between POS phagocytosis and the endocytic pathway. Although CME and CavMe mechanism of endocytic pathway has been a well-studied there remains limited understanding to CIE and other endocytic pathways. Our study offers unique insight into an equally understudied area in RPE biology and bridges this gap.

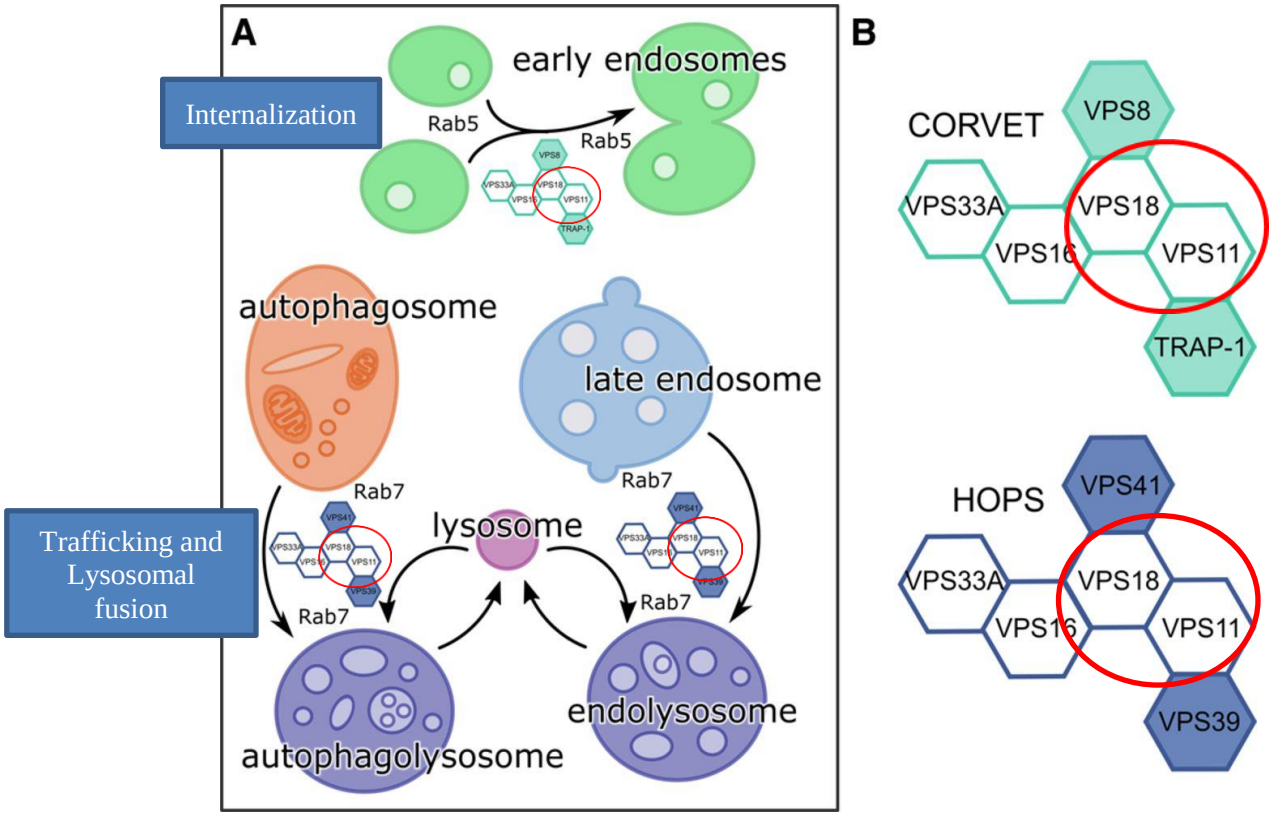

Illustration 1 COVERT & HOPS Complex Adapted from

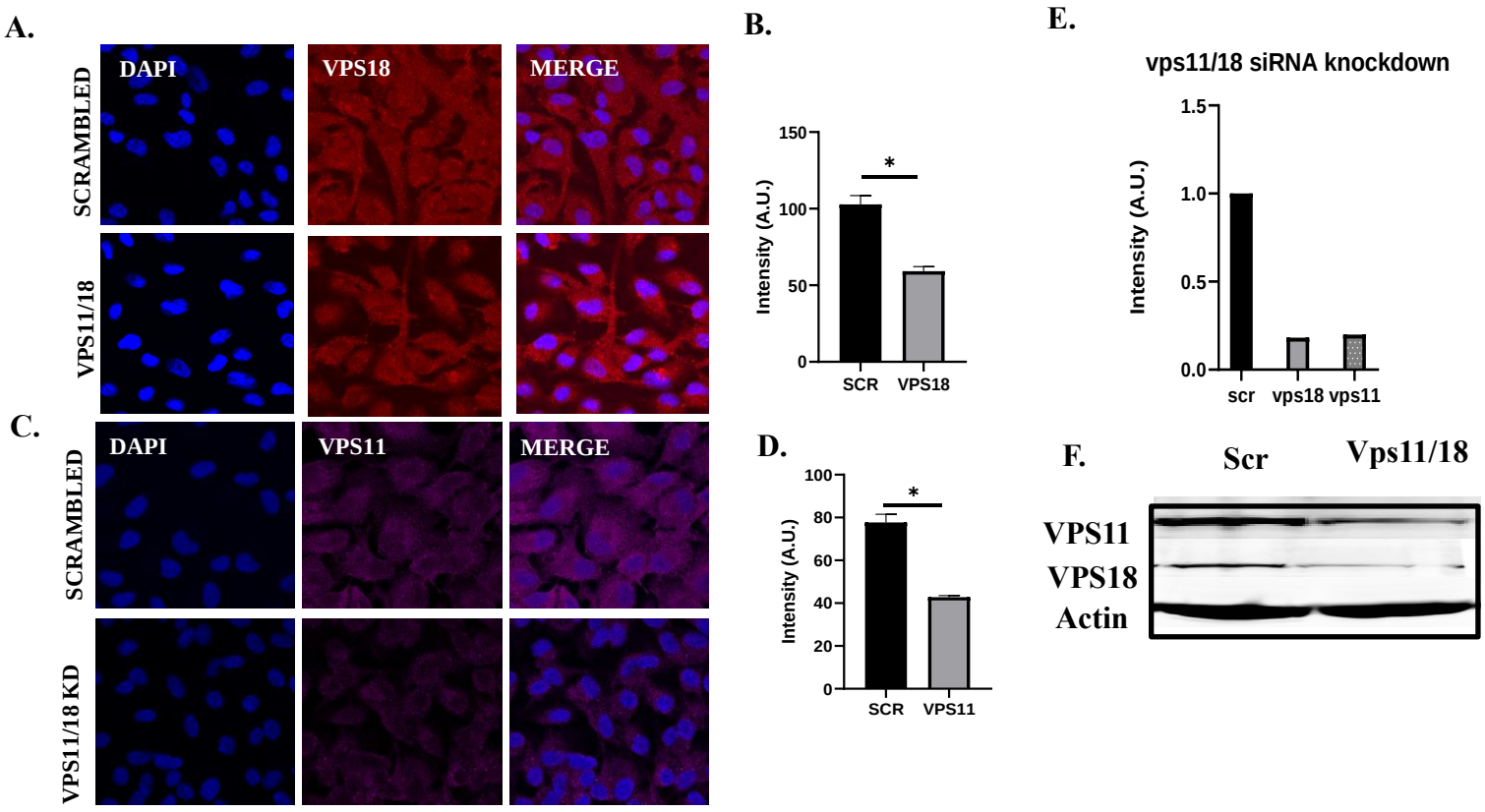

Figure 2.2 vps11 and vps18 combination siRNA knockdown

A. siRNA knockdown of vps18 in vps11/vps18 knockdown cells **B.** Vps18 intensity was significantly reduced in knockdown. **C**. siRNA knockdown of vps11 in vps11/vps18 knockdown cells. **D.** Vps11 intensity was significantly reduced in knockdown. **E**. vp11/vps18 knockdown efficiency in RPE cells. Quantification of (F). **F.** Western Blot of vps11 and vps18 knockdown in vps11/vps18 combination knockdown cells.

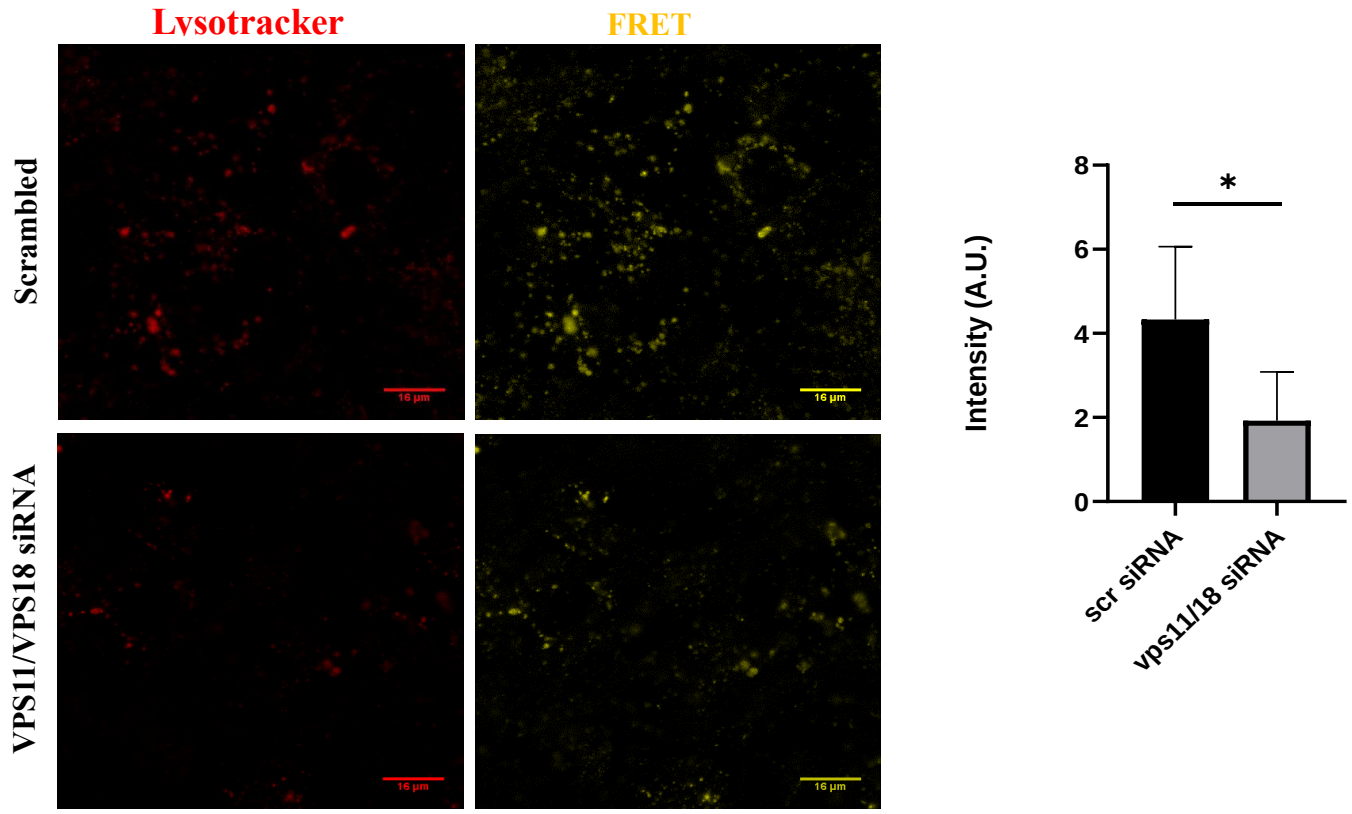

Figure 2.3 vps11/vps18 knockdown RPE cells have reduced FRET signal upon POS exposure

Vps11/vps18 knockdown cells were challenged with FITC- POS for 2h. FRET intensity was reduced in scrambled control cells compared to knockdown cells.

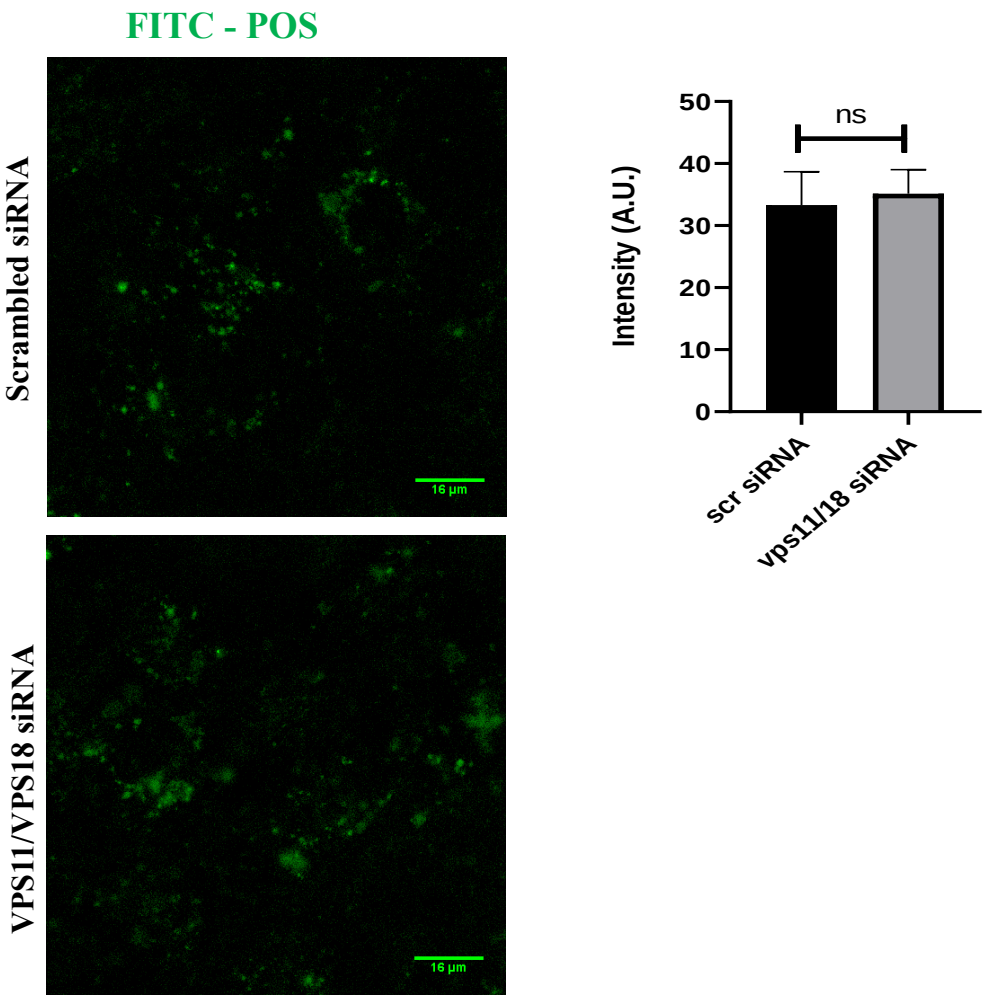

Figure 2.4 vps11/vps18 knockdown does not affect POS uptake

A. Vps11/vps18 combination knockdown cells challenged with FITC – POS for 2h. Representative image from different experiments (n=3) **B.** FITC-POS intensity was unchanged in knockdown cells compared to scrambled control.N = 3, Mean ± SEM, ns p >.05. Scale Bar 16µm

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Age – related macular degeneration (AMD), the leading cause of vision loss in the USA among adults 50 and older, is caused by loss of photoreceptor cells and retinal pigment epithelium (RPE) cells within the macula. RPE cells provide support to the retina by participating in a variety of tasks; the most important being, daily phagocytosis of shed photoreceptor outer segment (POS). Failure to clear shed POS contributes to build up of intercellular debris within the RPE and eventual formation of extracellular fatty protein deposits. Age related changes in RPE and retina can lead to decrease in efficiency of the clearance of shed POS. Since RPE phagocytosis contributes to retinal health, understanding the molecular mechanisms involved in this non – traditional phagocytic pathway is crucial for discovering new targets for treatment of retinal degeneration diseases like AMD. There remains a gap in knowledge concerning factors involved in trafficking of the POS to lysosome for degradation. To discover new factors involved in trafficking of POS, there must be quantitative methods of assessment. Conventional assays provide minimal information and do not offer distinct information about each stage of phagocytosis. Developing a quantitative assay to monitor trafficking of POS will allow molecular dissection of unknown factors that may be involved in intracellular trafficking of POS. I developed a quantitative fluorescence assay to monitor POS trafficking in RPE using Foster Resonance Energy Transfer (FRET). FITC or Fluorescein is used as FRET donor, to label the membrane of the POS. Lysosomes in immortalized human RPE cells, are labelled with lysosomal labeling dye, lysotracker (acceptor). Sensitized emission of the acceptor is detected, as FITC labelled POS are up taken by the RPE cells and reach the lysosome. Lysotracker labeled lysosomes in human RPE cells, undergo an energy transfer with FITC labeled POS when excited at 463nm; characteristic excitation wavelength of FITC. This assay allows for detection of POS trafficking to the lysosome in human RPE cells in culture. The novel assay offers a quantitative assessment of POS trafficking in RPE and allows molecular dissection of factors which are involved in the phagocytic pathway. This quantitative assay was then used to uncover vacuolar protein sorting associated proteins (VPS) 11 and 18 role in intracellular trafficking of POS. VPS11/18 knockdown in RPE cells produces aberrant trafficking of POS in RPE. The novel assay can be used to uncover other molecular machinery involved in the trafficking stage of POS phagocytosis.

University of Texas Medical Branch
Galveston, TX
Graduate Student
June 2015 – July 2016
PI: Dr. Krishna Bhat

An important step during nervous system development is the establishment of neuronal networks. Proper functioning of the nervous system depends on a precise wiring of neuronal networks. This requires pathfinding of axonal projections, controlled by multiple extracellular cues. Aberrant axon guidance has been linked to human diseases such as Corpus Callosum Dysgenesis and Horizontal Gaze Palsy with Progressive

Scoliosis. We use *Drosophila* as a model system in studying molecular pathways involved in axon guidance. *Drosophila* is an outstanding genetically tractable system that has been utilized to understand axon guidance. One of the major axon guidance signaling system is mediated by Slit and Robo pathway. This pathway generally mediates a repulsive axon guidance system. It has been well documented that Slit undergoes proteolytic processing yielding two fragments; a large C - terminal fragment and a smaller N - terminal piece. Little is known of the role of Slit processing in the function of this signaling pathway. We have generated transgenic *Drosophila* lines carrying full length, N and C terminal portions of the Slit protein. These transgenic lines will be used to determine their ability to rescue slit loss of function defects as well as if they have any gain of function effects in order to elucidate the functional significance of the two portions of Slit in the signaling pathway. We have also identified the presence processed and full length slit in the adult brain. Based on the presence of slit post development, we think that the protein has additional roles in the brain. Future genetic screens can be used to elucidate other players involved in the signaling of Slit developmentally and post-development and adult stages.

University of Texas Medical Branch
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January 2015 – April 2015
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Current research has produced immense information on the role of serotonin (5-HT) and its cognate receptors in substance use disorders. Specifically, dysregulation of the G protein-coupled receptor (GPCR) 5-HT_{2C} receptor (5-HT_{2C}R) system has been linked to relapse in cocaine use disorder. A deep understanding of 5-HT_{2C}R function and its causative role in neurobiology is critical in the process of design of new molecular therapeutic approaches to such disorders. One barrier to these efforts is the lack of knowledge of the macrocomplex associated with the 5-HT_{2C}R and how these protein partners influence the status of the system. The overall goal of the project is to isolate the 5-HT_{2C}R macrocomplex in its activated state in a neuronal cell line. The first step in reaching this goal is to ensure proper expression and functionality of 5-HT_{2C}R in the Neuro2A cells, a mouse neural crest-derived cell line that has been used to study neuronal differentiation and GPCR signaling pathways. Cell transfection and intracellular calcium release immunoassays were used to examine the functionality of the 5-HT_{2C}R in Neuro2A cells. The Neuro2A cell RNA profile was also determined by RNAseq method.

University of Texas Medical Branch
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Rotation Student
September 2014 – December 2014
PI: Dr. Yogesh Wairkar

Parkinson's disease (PD) is a neurodegenerative disease, which results in the loss of dopaminergic neurons and is characterized by the formation of α –synuclein oligomers accumulating into aggregates known as Lewy bodies. Previous studies have identified the toxic species of PD as the oligomeric α –synuclein. The study's main goal was to establish a model which can be useful in further understanding how the formation or accumulation of α –synuclein oligomers affects the synapses and causes neurodegeneration in *Drosophila*. In order for a model to be useful there must be confidence that the features observed in the model can closely mimic characteristics of the human disease prior to initiating further study. α –synuclein expression has been experimentally shown to closely mimic some aspects of PD pathology. The *Drosophila* model should contain α –synuclein oligomers. To accomplish this *Drosophila* dopaminergic driver, Th-gal4 was used to drive the expression of human α –synuclein in dopaminergic neurons. Th-gal4 specifically drives expression in dopaminergic cells in larval CNS and adult brains when it is introduced in the *Drosophila* genome. The pattern of expression was assessed by using the dopaminergic driver in conjunction with a UAS-GFP. Immunohistochemistry confirmed the driver properly expresses in dopaminergic neurons. Immunoblotting experiments were done to confirm that α –synuclein oligomers are expressed in these *Drosophila* lines.

University of Texas Medical Branch
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Research Volunteer
June 2013 – August 2014
PI: Yogesh Wairkar

Engaged in research relating to molecular mechanism related to synaptic dysfunction and neurodegenerative diseases. Skills acquired included western blot, immunohistochemistry, RT- PCR, and high resolution confocal imaging

PRESENTATIONS:

University of Texas Medical Branch
PhD Oral Proposal Defense
November 20th, 2017
"Deciphering underlying mechanisms of photoreceptor outer segment degradation: involvement of intracellular trafficking"

University of Texas Medical Branch
Cell Biology Symposium
May 4, 2016
"A fluorescence based quantitative assay to monitor photoreceptor outer segment trafficking in retinal pigment epithelium"

University of Texas Medical Branch

Advanced Academic Skills Course Proposal Presentation
April 19, 2016
"Deciphering underlying mechanisms of photoreceptor outer segment degradation: involvement of intercellular trafficking"

University of Texas Medical Branch
Cell Biology Symposium
May 4, 2015
"Axon guidance molecules post-developmental expression"

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Neuroscience Graduate Program Annual Student Seminar
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ACADEMIC SERVICE:
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Society of Cell Biology, UTMB
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HONORS, AWARDS & FELLOWSHIPS:
Society of Cell Biology Service Award – April 2019
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PUBLICATIONS IN -PREPARATION:

Egbejimi, A., Cai, J., Chen, Y. Novel assay to monitor photoreceptor outer segment trafficking in retinal pigment epithelium

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B. Natarajan R, Barber K, Buckley A, Cho P, **Egbejimi A**, Wairkar YP. 2015. Tricornered Kinase Regulates Synapse Development by Regulating the Levels of Wiskott-Aldrich syndrome Protein. PLoS ONE.;10(9):e0138188. doi:10.1371/journal.pone.0138188.

C. David J. Durgan, Michael W. S. Moore, Ngan P. Ha, Oluwaseun Egbejimi, Anna Fields, Uchenna Mbawuike, **Anu Egbejimi**, Chad A. Shaw, Molly S. Bray, Vijayalakshmi Nannegari, Diane L. Hickson-Bick, William C. Heird, Jason R. B. Dyck, Margaret P. Chandler, Martin E. Young. 2007 Circadian rhythms in myocardial metabolism and contractile function: influence of workload and oleate. American Journal of Physiology - Heart and Circulatory Physiology Oct, 293