THE OCULAR SURFACE CONTROL OF BLINKING, TEARING AND SENSATION

Ziwei Wu

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Doctoral Committee

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Chairperson, Carolyn Begley, OD, MS

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Arthur Bradley, PhD

_____

Nicholas Port, PhD

_____

Pete Kollbaum, OD, PhD

_____

Trefford Simpson, DipOptom, PhD

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Ziwei Wu

THE OCULAR SURFACE CONTROL OF BLINKING, TEARING AND SENSATION

Dry eye is a common condition that affects millions in the US (Schaumberg, Sullivan et al. 2003; Schaumberg, Dana et al. 2009) and worldwide.(McCarty, Bansal et al. 1998; Uchino, Nishiwaki et al. 2011) It is considered to be a multifactorial disease of the tear film and ocular surface and is associated with symptoms of ocular discomfort and visual disturbance.(2007) Low blink rate has been identified as a potential risk factor for the development of dry eye because it can result in increased evaporative loss from the tear film.(Tsubota and Nakamori 1995; Nakamori, Odawara et al. 1997; 2007; Ousler, Hagberg et al. 2008; Himebaugh, Begley et al. 2009) Failure of tear secretion has also been recognized as one of the main factors for dry eye development, characterized as low tear volume and slow tear turnover rate.(2007) Both factors in turn may lead to increased tear film hyperosmolarity (Liu, Begley et al. 2009) and instability,(Liu, Begley et al. 2006) which are considered core mechanisms of dry eye.(2007) In the natural condition, the ocular surface is mainly protected by blinking and tear secretion in that the newly secreted tears flow into the upper and lower meniscus and the blink spreads the new tear film from the meniscus to the ocular surface.(Oyster 1999) Therefore, the ocular surface control over blinking and tear secretion is important in the etiology of the dry eye condition.

In this proposal, we develop a laboratory model (Figure 1) using human subjects to test how input from the ocular surface affects both blinking and tear secretion. We hypothesize that ocular surface stimuli will activate corneal receptors to signal a high blink rate, reflex tear secretion and ocular sensations of discomfort. These probably act together for the purpose of preventing ocular damage. These results will help us to understand the manner in which the ocular surface responds to adverse stimuli, which may ultimately lead toward further development of treatments or methods in dry eye patients.

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CHAPTER I

INTRODUCTION

Purpose of Thesis

Dry eye is a common condition that affects millions in the US (Schaumberg, Sullivan et al. 2003; Schaumberg, Dana et al. 2009) and worldwide.(McCarty, Bansal et al. 1998; Uchino, Nishiwaki et al. 2011) It is considered to be a multifactorial disease of the tear film and ocular surface and is associated with symptoms of ocular discomfort and visual disturbance.(2007) Low blink rate has been identified as a potential risk factor for the development of dry eye because it can result in increased evaporative loss from the tear film.(Tsubota and Nakamori 1995; Nakamori, Odawara et al. 1997; 2007; Ousler, Hagberg et al. 2008; Himebaugh, Begley et al. 2009) Failure of tear secretion has also been recognized as one of the main factors for dry eye development, characterized as low tear volume and slow tear turnover rate.(2007) Both factors in turn may lead to increased tear film hyperosmolarity (Liu, Begley et al. 2009) and instability,(Liu, Begley et al. 2006) which are considered core mechanisms of dry eye.(2007) In the natural condition, the ocular surface is mainly protected by blinking and tear secretion in that the newly secreted tears flow into the upper and lower meniscus and the blink spreads the new tear film from the meniscus to the ocular surface.(Oyster 1999) Therefore, the ocular surface control over blinking and tear secretion is important in the etiology of the dry eye condition.

Controls over spontaneous blinking, which include all but voluntary or reflex blinks,(Evinger, Manning et al. 1991) remain controversial.(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Naase, Doughty et al. 2005) Blink rate is known to be highly variable and depends on many factors, including cognitive state,(Bentivoglio, Bressman et al. 1997; Doughty 2001; Hirokawa, Yagi et al. 2004) ocular surface input,(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Doughty, Naase et al. 2009) central dopamine level (Karson 1983; Barbato, De Padova et al. 2007; Agostino, Bologna et al. 2008) and inter-subjects variation.(Freudenthaler, Neuf et al. 2003; Doughty and Naase 2006) Visual tasks requiring concentration, such as playing a computer game or reading are known to slow the blink rate,(Acosta, Gallar et al. 1999; Doughty 2001; Himebaugh, Begley et al. 2009; Cardona, Garcia et al. 2011) sometimes markedly,(Schlote, Kadner et al. 2004) whereas stimulation or irritation of the ocular surface can increase the blink rate.(Nakamori, Odawara et al. 1997; Himebaugh, Begley et al. 2009) The apparent excitatory input of the ocular surface sensory mechanisms on blinking is of special interest due to its putative effect in dry eye,(Palakuru, Wang et al. 2007) where sensory input from the unstable tear film(Liu, Begley et al. 2009) and corneal sensory nerve damage or functional changes may be expected to affect blinking.(Peshori, Schicatano et al. 2001; Belmonte, Acosta et al. 2004; Toshida, Nguyen et al. 2007; Situ, Simpson et al. 2008; Kaminer, Powers et al. 2011). Despite the importance of blinking, the effect of varying the level of ocular surface stimulation on blink has not been explored under controlled experimental condition.

For tear secretion, most studies focus on the neural pathway of lacrimal reflex arc (Stern, Beuerman et al. 1998; Stern, Gao et al. 2004; Stapleton, Marfurt et al. 2013) and

the cellular mechanisms of water and protein secretion. (Dartt, Moller et al. 1981; Hodges and Dartt 2003; Dartt 2004) Reflex tear secretion is triggered from the irritation of the ocular surface, along with other factors.(Rohatgi, Gupta et al. 2005; Murube 2009) Afferent nerve fibers from the corneal receptors go through the trigeminal nerve, projecting on the brainstem. The efferent nerve fibers go through the ophthalmic nerve and finally control the lacrimal glands. The water and protein component in reflex tears are modulated by both parasympathetic and sympathetic neural transmitters. (Dartt 2004; Dartt 2009) Despite the importance of tear secretion on maintaining ocular surface health, few studies examine tear dynamics in human subjects while varying ocular surface stimulation.

In this proposal, we develop a laboratory model (Figure 1) using human subjects to test how input from the ocular surface affects both blinking and tear secretion. We hypothesize that ocular surface stimuli will activate corneal receptors to signal a high blink rate, reflex tear secretion and ocular sensations of discomfort. These probably act together for the purpose of preventing ocular damage. These results will help us to understand the manner in which the ocular surface responds to adverse stimuli, which may ultimately lead toward further development of treatments or methods in dry eye patients. We will constitute three experimental chapters in this thesis:

1. **To compare the effects of concentrating on a visual task and a mild ocular surface air stimulation on blink behavior and tear film stability.** We hypothesize that 1) concentrating on a visual task and having a mild surface stimulation will have opposite effects on the rate, fullness and duration of the blink. 2) Tear film instability

will be correlated with blink parameters, since it will stimulate the underlie neurons, sending signals to the blink center. To test this, we will:

a. **Measure the blink frequency and detailed blink parameters, including amplitude, velocity and duration, under different conditions.** A computer game will be provided to require visual attention, and a mild air flow will be delivered from an electric fan to stimulate the ocular surface. *We hypothesize that concentration on a visual task will decrease the blink rate, fullness and duration to minimize the lid interruption to vision, whereas having a mild ocular stimulation will increase the blink rate, fullness and duration to protect the eye from potential damages.*

b. **Quantify the tear film instability and determine whether changes in tear film are associated with altered blinking parameters.** We will measure the area of tear breakup to quantify the tear film instability and correlated it with each blink parameter. *We hypothesize that the stimulus will increase tear film evaporation and decrease tear film stability, which will provide a stimulus for altering blink behaviors.*

2. **To examine the responses of blink and sensation in the presence of an increasing ocular surface stimulation while controlling task concentration.** We hypothesize that 1) both blink frequency and subjective sensations will increase with surface stimulation. 2) Blink and subjective sensations will be correlated since they are sharing the same inputs from the ocular surface. To test this, we will:

a. **Measure the blink frequency and subjective sensation under different air stimuli while the cognitive state is controlled.** We will use a customized

machine similar as esthesiometer to devise a set of air stimuli with different intensities that mainly focus on the ocular surface. The blink frequency (blink rate and interblink interval) and subjective sensations to these stimulations will be measured. *We hypothesize that increasing surface stimuli will increase blink frequency and sensation and there should be an air intensity that significantly change the blink response (blink increase threshold) from its baseline.*

b. **Determine the correlation between blink and ocular sensation.** Blink frequency and sensation grades will be correlated. *We hypothesize that blink and sensory are correlated because they are sharing the same inputs from ocular surface.*

3. **To examine the response of tear secretion, blink and their interaction in the presence of increasing ocular surface stimuli while cognitive state is controlled.** *We hypothesize that an increased frequency of blinking and tear secretion will occur with increasing stimuli to the ocular surface to protect the ocular surface. We expect tearing and blinking to be correlated in some manner because the initial input for each arises from the ocular surface.* To test this, we will:

   a. **Develop new technique and metrics to quantify the tear secretion over time.** We will use fluorescein to visualize the lower tear meniscus, and the tear meniscus height and fluorescein concentration will be measured and calculated over time. The fluorescein turnover rate between blinks will be calculated to quantify the tear secretion separating from blinks.

   b. **Measure the response of blink frequency and tear secretion simultaneously to varied ocular surface stimulation.** *We hypothesize that increasing ocular*

surface stimulation will induce tear secretion and blinking which will increase the tear turnover rate.

c. **Determine the relationship between blinking and tear secretion with ocular surface stimulation.** *We hypothesize that these outputs are correlated since they both arise from stimulation of the ocular surface.* However, the timing and intensity of the responses may differ from each other since because they may be initially stimulated by different neural subclasses on the ocular surface and their output pathways are not the same.

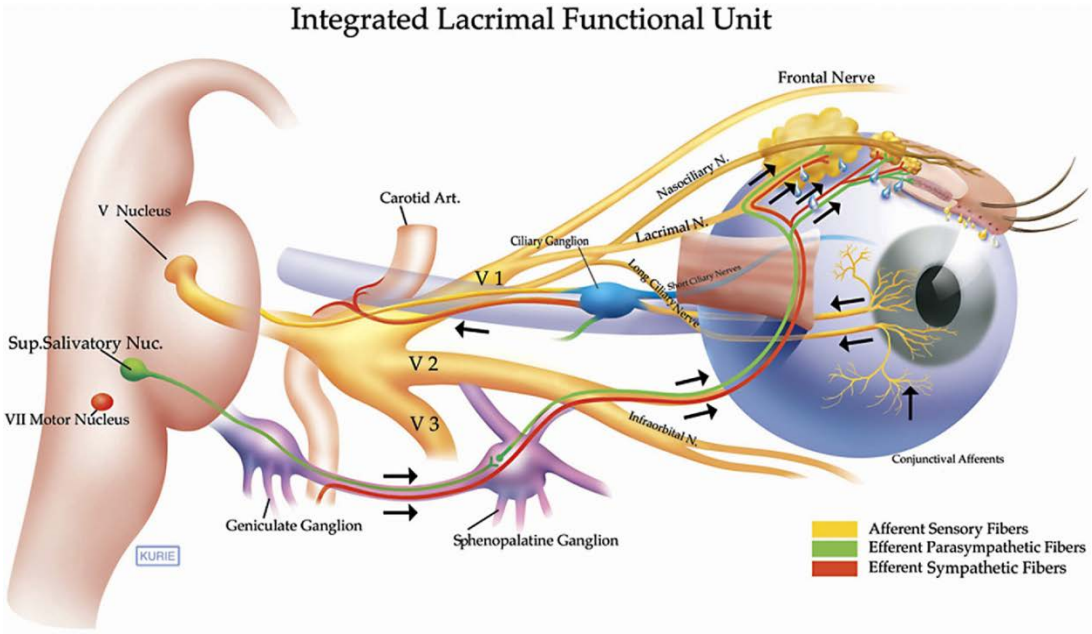

Figure 1: The Schematic representation of the lacrimal functional unit.(Pflugfelder 2011) The receptors on the ocular surface detect the external stimulus, sending signals to the brainstem through the trigeminal nerve. The efferent fibers from superior salivatory nucleus innervate the lacrimal gland, and efferent fibers from facial (VII) motor nucleus innervate the orbicularis muscles for trigger a blink.

Significance of Dry Eye Disease

Dry eye disease (DED) is a common condition. Based on studies with large sample size, it has been estimated that about 3.32 million women and 1.69 million men (Schein, Munoz et al. 1997; Moss, Klein et al. 2000; Schaumberg, Sullivan et al. 2003; Schaumberg, Dana et al. 2009), who are 50 years and older, have dry eye in America. More than ten million Americans have mild to moderate dry eye symptoms when they are wearing contact lens or living in a low humidity environment. The financial cost of dry eye is a high burden for patients, including the health care system utilization, office visits and prescription medications (Reddy, Grad et al. 2004).

The 2007 Dry Eye Workshop defined dry eye as "a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tears film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface". Two major subclasses of DED have been identified as Figure 2: aqueous tear-deficient dry eye (ADDE) and evaporative dry eye (EDE). ADDE is mainly due to the decreasing lacrimal secretion. In this case, even though the evaporation rate may be still normal, the overall tear volume and thickness is decreased with low liquid delivered from lacrimal gland. EDE is mainly due to the massive evaporation over the ocular surface, that the rate of evaporation is higher than the secretion. The causes of the EDE subdivide to intrinsic and extrinsic influence. The intrinsic factors include low blink rate, wide eye aperture and the lipid layer dysfunction. The extrinsic factors include living in low humidity, high wind velocity and hot environments. Dry eye can be initiated by any subclass; they are not

mutually exclusive. For example, dry eye may cause by meibomian dysfunction so that less lipid is secreted into the tears, leading to the EDE. The EDE will then initiates the inflammation over the ocular surface and interrupt the normal corneal nerve function, decreasing the tear secretion (ADDE). On the other hand, if the initial tear secretion is low (ADDE), even a normal evaporation will lead to hyperosmolarity or inflammation on the ocular surface, which might damages the lipid function or secretion, resulting in EDE.

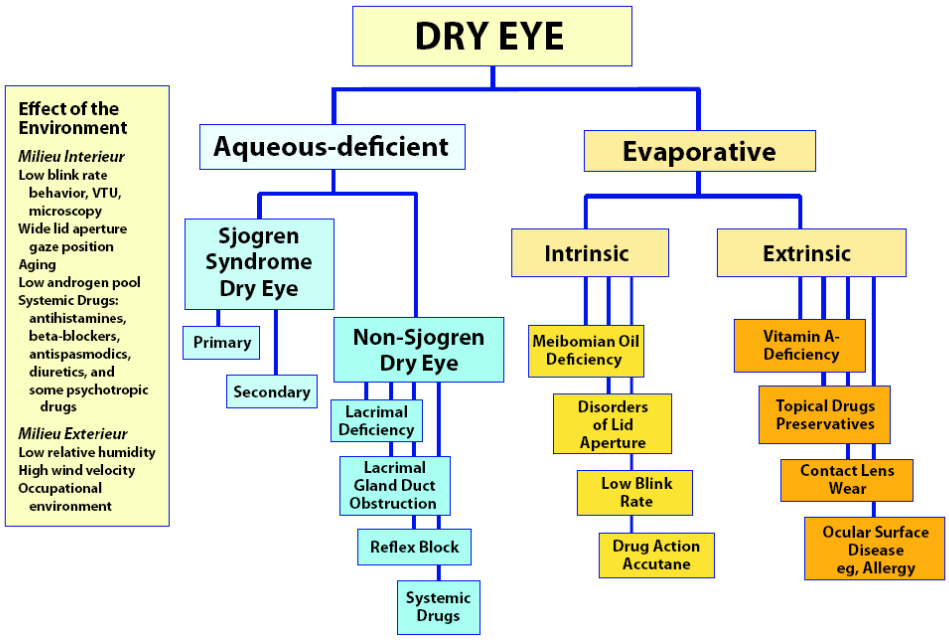

Figure 2: Diagram of major etiological causes of dry eye. (2007) The left box shows the possible risks for development of dry eye, including the internal and external factors.

Even though dry eye affects millions, the cause of DED remains unclear and there is little agreement on which combination of tests should be used to define the disease (Hay, Thomas et al. 1998; McCarty, Bansal et al. 1998; van der Worp, De Brabander et

al. 2003). Several risk factors are found for DED (Table 1), such as aging or female gender (Tan, Keay et al. 2003; Szczesna 2011), but no direct evidence shows how these risk factors contribute to the development of the condition (Rucker 2011). Another challenge for dry eye studies is the apparent lack of correlation between patients' ocular symptoms and clinical signs of dry eye, perhaps because the tests do not measure the cause of the symptoms (Bjerrum 1996; Hay, Thomas et al. 1998; van der Worp, De Brabander et al. 2003). Many clinical tests show poor repeatability which adds to the natural variability of dry eye disease. Finally, the ocular symptoms reported by patients are subjective descriptions that can be influenced by many factors, including pain thresholds and a desensitized ocular surface with age or other eye diseases. Symptom questionnaires are among the most repeatable and commonly used methods to reveal the dry eye condition (Koh, Maeda et al. 2006; Liu, Begley et al. 2006; Ounnoughene, Benhatchi et al. 2006; Wang, Aquavella et al. 2006). The poor correlation between clinical signs and symptoms of dry eye has led to difficulties in devising treatments for the condition due to problems in proving treatment efficacy.(Nichols, Nichols et al. 2004)

Level of Evidence		
Mostly consistent*	**Suggestive†**	**Unclear‡**
Older age	Asian race	Cigarette smoking
Female sex	Medications	Hispanic ethnicity
Postmenopausal estrogen therapy	Tricyclic antidepressants	
Omega-3 and Omega-6 fatty acids	Selective serotonin reuptake inhibitors	Anti-cholinergics
Medications	Diuretics	Anxiolytics
Antihistamines	Beta-blockers	Antipsychotics
Connective tissue disease	Diabetes mellitus	Alcohol
LASIK and refractive excimer laser surgery	HIV/HTLV1 infection	Menopause
Radiation therapy	Systemic chemotherapy	Botulinum toxin injection
Hematopoietic stem cell transplantation	Large incision ECCE and penetrating keratoplasty	
	Isotretinoin	Acne
Vitamin A deficiency	Low humidity environments	Gout
Hepatitis C infection	Sarcoidosis	Oral contraceptives
Androgen deficiency	Ovarian dysfunction	Pregnancy

* Mostly consistent evidence implies the existence of at least one adequately powered and otherwise well-conducted study published in a peer-reviewed journal, along with the existence of a plausible biological rationale and corroborating basic research or clinical data.

† Suggestive evidence implies the existence of either: 1) inconclusive information from peer-reviewed publications or 2) inconclusive or limited information to support the association, but either not published or published somewhere other than in a peer-reviewed journal

‡ Unclear evidence implies either directly conflicting information in peer-reviewed publications, or inconclusive information but with some basis for a biological rationale

Table 1: Major etiological causes of dry eye (Ishida, Kojima et al. 2005)

Dry eye is recognized as a disturbance of the lacrimal function unit (LFU), which includes the lacrimal glands, ocular surface (cornea, conjunctiva and meibomian glands), eyelids and the sensory and motor nerves that connect them (Walinder, Ernstgard et al. 2005). An important aspect of the unit is the sensory signals which arise from the ocular surface. The signals from the ocular surface may maintain basal tear levels and trigger spontaneous blinking under normal conditions (Mutch 1944). A defect in any part in the LFU can interrupt tear film stability, leading to dry eye symptoms (DEWS Report, 2007). In this proposal, I seek to understand the ocular surface controls over blinking, tearing and subjective sensation and how they interact with each other to protect the ocular surface.

General Introduction to the Ocular Surface

1. Cornea

The cornea has an aspheric surface, which plays a big role in the refractive process. From the front view, the average diameter of the cornea is 12.6mm horizontally, and 11.7mm vertically. The radius of the curvature and the thickness of the cornea increases from the center to the peripheral area. The central third of the cornea surface, with the corneal apex at its center (4mm in diameter), is called the optical zone, and is close to spherical. Since there is a large refractive index difference between the cornea and the air, the front ocular surface provides the strongest refractive power (approximately +48 diopters) to converge the light onto the retina. The cornea and tear film contribute about 40 of the 60 diopters of the whole visual system (Oyster, 1999). In normal conditions, more than 90% of the light (wavelength from 400 to 740 nm) can go through the cornea without scattering and absorbing (Farrell, McCally et al. 1973). The reasons for corneal transparency will be discussed later (in the corneal stroma section).

As the most anterior structure of the eye, the cornea has a specific arrangement to maintain its functions. The cornea is transparent. Five layers have been identified within the cornea (as Figure 3), and they each contribute differently to corneal function.

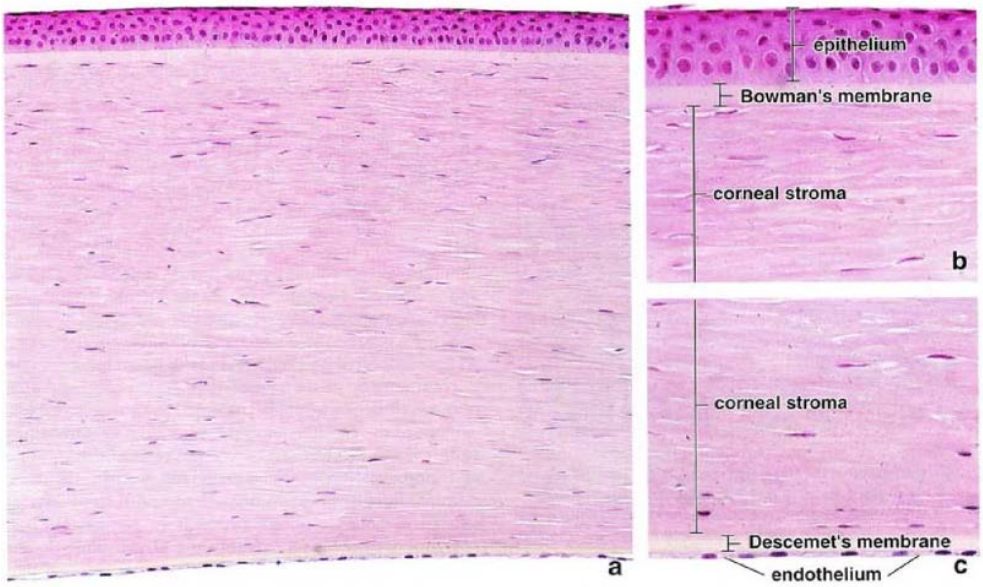

Figure 3: Diagram of cornea structure by using photomicroscope. Five layers have been identified. Image adapted from Internet (http://quizlet.com/11039887/histology-eye-notable-figures-flash-cards/).

1.1 Epithelium

The epithelium, which is the most anterior layer of the cornea, contains three sub-layers: the basal cell layer, the wing cell layer and the squamous cell layer (Figure 4). The lowest layer of the epithelium is comprised of basal cells which are the newest cells, and have a square-like shape. These form one-cell layer just on top of the basal membrane and Bowman's layer. Above the basal cells, there are about two to three layers of wing cells, which are so called because several wing-like, irregular projections extend from the cell bodies. The most anterior two to three layers contain squamous cells, which have a flattened shape and microvilli extending into the tear film. The microvilli along the apical

surface of the squamous cells significantly increase the interface between epithelium and tear film, stabilizing the tear film over the ocular surface. In addition, the squamous cells secrete mucin, which will be discussed in detail in the tear film section.

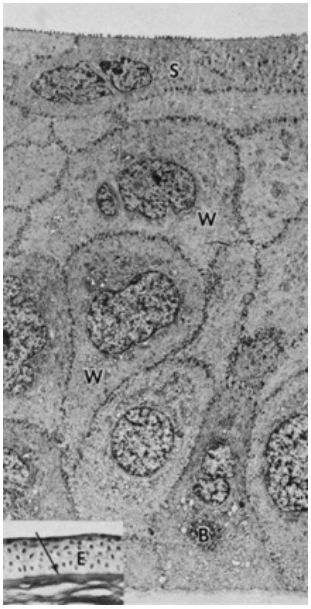

Figure 4: electron micrograph of the corneal epithelium. The shapes of cells in different layers are changed from the bottom to the top. The microcilli are found at the very top of the epithelium to increase the interface between cornea and tear film. Abbreviation: S, squamous cell; W, wing cell; B, basal cell. Image adapted from Internet (http://www.oculist.net/downaton502/prof/ebook/duanes/pages/v7/v7c008.html)

The epithelium is renewed by a constant cycle, which is called the X, Y, Z theory of corneal epithelial maintenance (Thoft and Friend 1983). This cycle promotes cell differentiation from the basal to the squamous cells, and keeps regenerating the corneal epithelium (as Figure 5). The source of the new basal cells is the limbal epithelium,

which is located at the transition zone between cornea and sclera. The limbal cells differentiate into basal cells, and move toward the corneal center, forming the basal cell layer. During this process, the basal cells in the central area are pushed up and the shapes of the basal cells are transformed from square to an irregular shape. At this point, the basal cells become wing cells, which slowly advance anteriorly until they reach the most superficial apical layer, forming the squamous cell layer. The squamous cells eventually slough off the ocular surface with tear film flow and the motion of the eyelid. They are washed into the tear film, and drain out through the puncta. The corneal epithelium is maintained by a balance of cell shedding, basal cell division and renewal of basal cells by centripetal migration of new basal cells originating from the limbal stem cells. This whole process normally takes 7 days (Hanna, Bicknell et al. 1961).

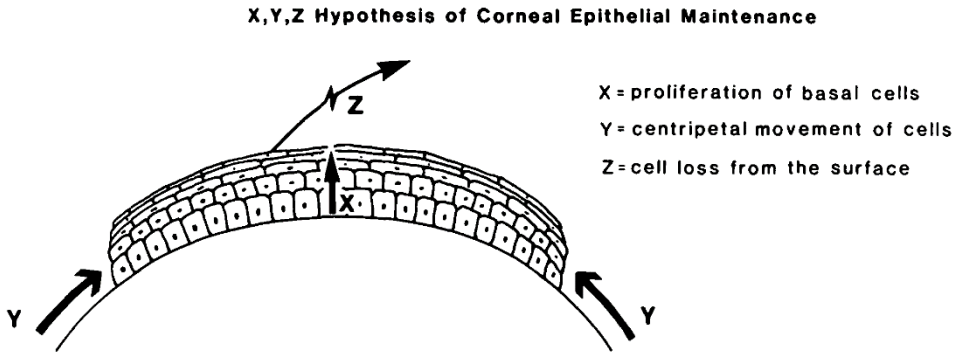

Figure 5: The X, Y, Z theory of epithelium maintainance (Thoft and Friend 1983).

The functions of the corneal epithelium include forming a barrier between the inside of the eye and the outside environment, communicating between cells and layers,

and adhering the epithelial sublayers to each other and the layers below. As Figure 6 shows, different cell junctions have been found within the epithelial layer.

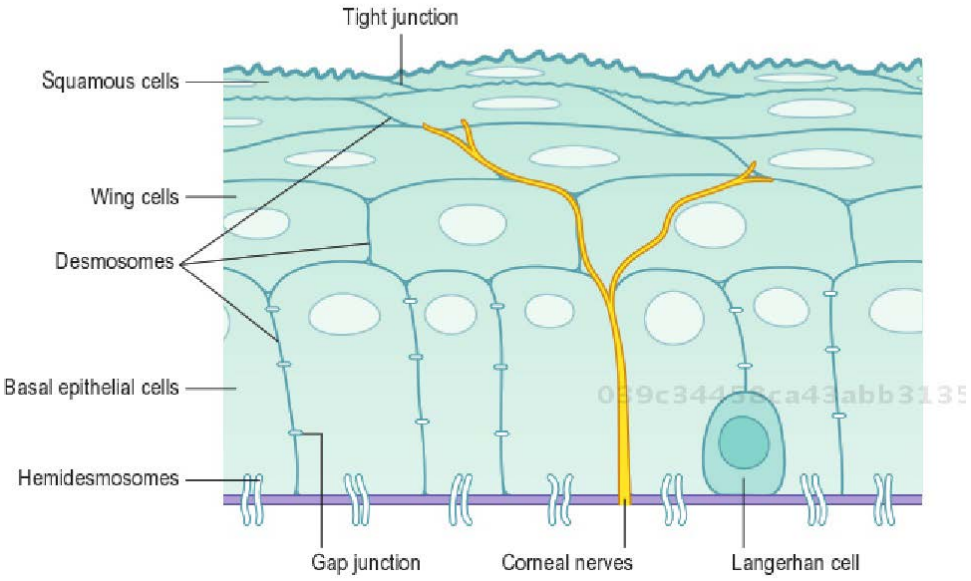

Figure 6: Cell junctions within the corneal epithelium (Levin and Kaufman 2011)

The squamous cells are bound to each other and to the underlying wing cells mainly by desmosomes. The basal cells are bound to the basement membrane by hemidesmosomes (Gipson, Grill et al. 1983), and there are desmosomes between the adjacent basal cells. These two junctions (the desmosome and the hemidesmosome) hold all the cells in the epithelium together, contributing to the rigidity and stability of both cells and tissues. (Levin and Kaufman 2011) Another two important connections are gap junctions and tight junctions. The gap junctions are found between the basal cells. They allow exchanges of small molecules and ions between adjacent cells, so that cells can communicate with each other. The gap junctions also exist between the cell layers,

especially between basal and wing cell layers, which suggests that gap junctions might be involved in transferring cells differentiation signals (Wiggert, Van Horn et al. 1982). Tight junctions are found between the superficial squamous cells (McLaughlin, Caldwell et al. 1985). Since the tear fluid covers the ocular surface, it is critical to prevent the water from flowing into the cornea. The zonula tight junctions, which are found in the top layers of squamous cells, encircle squamous cells to completely close off the extracellular space from surface liquids. Another type of tight junction, called spot tight junction, exists in the lower layers of squamous cells. It connects the adjacent cells, closing off the interspace between squamous cells. These two tight junctions block fluid from extracellular spaces and restrict small molecules from passing through the cells and intercellular space.

1.2 Bowman's Layer

Bowman's layer is a dense sheet located between the epithelial basement membrane and the anterior stroma (as Figure 7). It is described as an acellular sheet, but its function remains unclear. It is constructed of randomly arranged types I, II, V and VII collagen fibrils, which are interwoven with each other, forming a dense layer (Oyster, 1999). Some clinicians have speculated that Bowman's layer acts as a corneal ligament to maintain the corneal structure, since the collagen VII is related to anchoring fibrils of the epithelium (Tisdale, Spurr-Michaud et al. 1988). Others have suggested that the layer may serve as a barrier to inhibit invading viruses. Since the viruses need cells to propagate and spread, this acellular sheet might act as barrier to virus propagation.

However, there is no convincing data to support above hypothesis, and a viral infection can occur on a patient with a complete Bowman's layer. Therefore, the function of Bowman's layer is not clear (Wilson and Hong 2000).

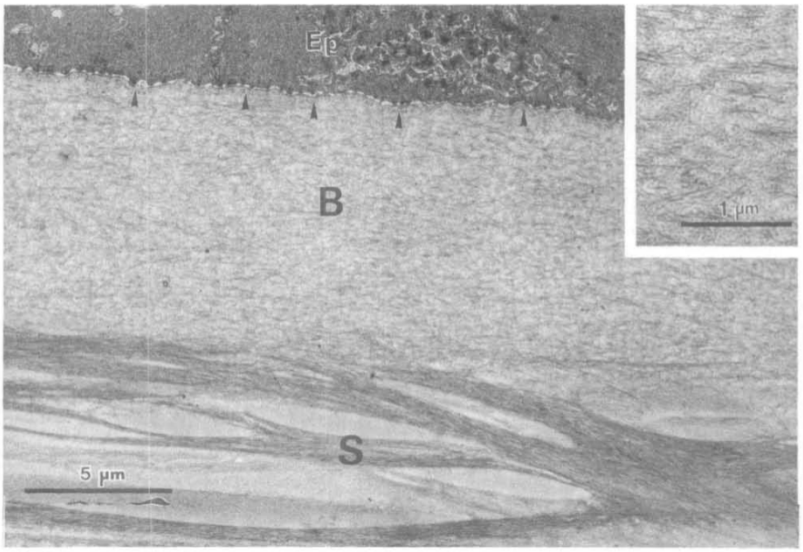

Figure 7: Diagram of the cross-section of the human cornea with the Bowman's layer. The inset image (top right) is a high magnification figure, showing the collagen fibrils in the Bowman's layer (Komai and Ushiki 1991). Abbreviation: Ep, epithelium; B, Bowman's layer; S, stroma.

1.3 Stroma

The stroma is the thickest part of the cornea, making up more than 90% of the whole corneal thickness (Komai and Ushiki 1991). It is mainly an extracellular matrix (ECM) comprised of collagen fibrils, proteoglycans and keratocytes (Cogan 1951). The

most important character of the stroma is transparency, which allows most of the incident visible light to go through the cornea without scattering.

The stroma is made of collagen fibrils with similar size and character. The biggest component of the stroma is collagen. Each collagen molecule contains three amino acid chains, which spiral around each other, forming a triple helix (Oyster, 1999). In the cornea, around 90% of the total collagen is type I (Newsome, Gross et al. 1982), which connect with each other at their end, forming long parallel molecules called fibrils. The collagen fibrils are similar in diameter and very regularly spaced (Maurice 1957). They run completely across the cornea, parallel to the ocular surface, forming bundles of collagen fibers called lamellas (as Figure 8). Each lamella is about 2.0 μm thick and 9 to 260 μm wide and there are approximately 200 to 500 lamellas in the stroma (Oyster, 1999). Since most collagen is type I, the fibrils are quite homogeneous, as well as similar in structure.

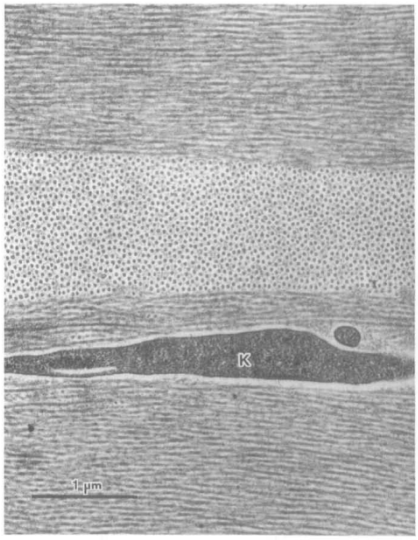

Figure 8: Diagram of cross-section of the corneal stroma lamellae. The fibrils have similar size and uniform interspace and they run in the same direction within each lamella (Komai and Ushiki 1991)

It is hypothesized that corneal transparency is due to the regular spacing between lamellas of collagen fibers. With the same interspace between fibrils, light scattering by individual fibril is canceled by the scattered light from adjacent fibrils, so that the light goes only in the forward direction (Maurice 1957). In order to maintain corneal transparency, the distance between the fibrils needs to be uniform and less than one half of the wavelength of visible light (Kaufman, 2003). This point could be supported by observing corneal transparency loss in certain diseases. Corneal edema involves increased fluid within cornea, which interferes the regular collagen fibril arrangement, leading to transparency loss (Figure 9). The swelling increases the distance between collagen fibrils and produces a more irregular spacing than under normal conditions. The loss of transparency increases as the amount of corneal swelling increases (Farrell, McCally et al. 1973). Further support for this theory is found in the scleral structure. In the sclera, the diameters and the space between collagen fibrils varies greatly and blood vessels and nerves are located between scleral fibrils. There is no regular spacing within its structure, leading to light scattering and an opaque structure (Figure 10).

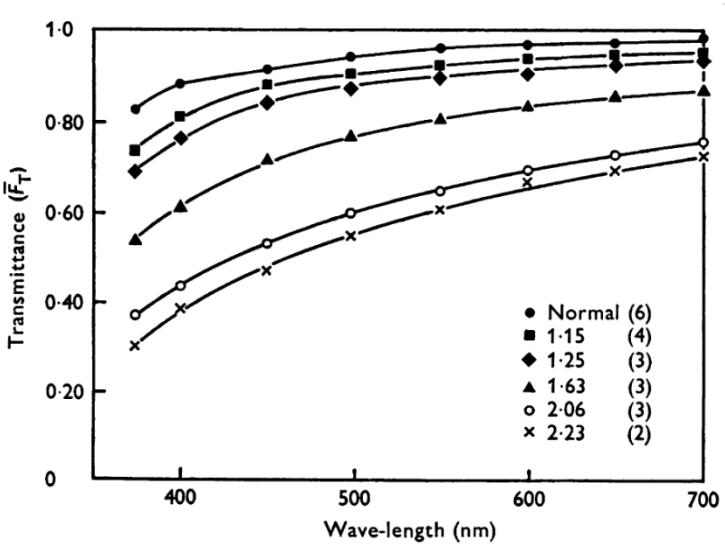

Figure 9: This figure shows the fraction of light transmitted as the function of wave-lengths. Different markers represent different corneal thicknesses. The thickest cornea has the lowest light transmittance, which means corneal swollen decreases the corneal transparency. (Farrell, McCally et al. 1973)

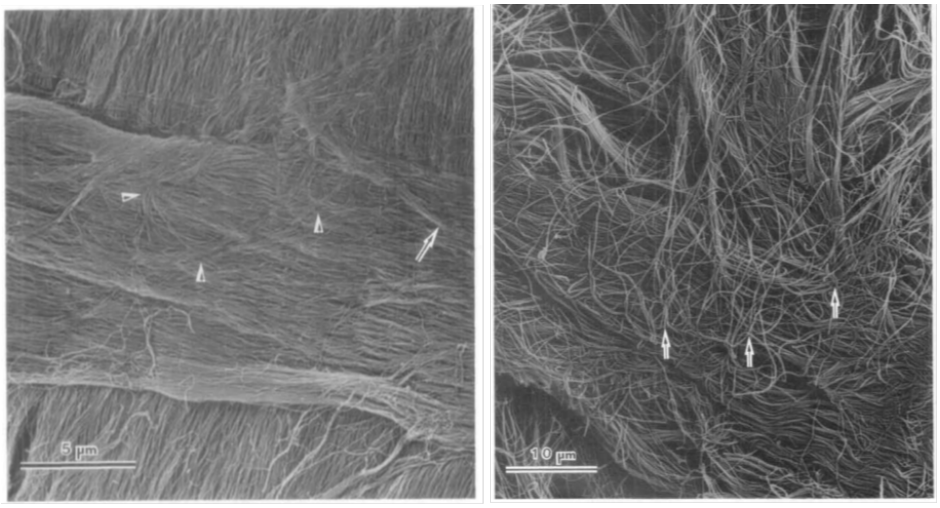

Figure 10: Both figures are high magnification images of lamella. The left is the lamella in the cornea, the right is in the sclera. The cornea has much more organized fibrils than that in the sclera (Komai and Ushiki 1991)

Proteoglycans in the extracellular matrix help maintain the regularity of the fibrils and give the cornea the ability to regulate its hydration. In order to maintain the regular spacing, proteoglycans existing between the corneal fibrils regulate the water content in the cornea, and the size of the fibrils. A proteoglycan contains a core protein chain to which many glycosaminoglycans are attached (GAGs) (Figure 11). In the cornea, there are two types of GAGs: dermatan sulfate and keratin sulfate. They attach to the core proteins, forming decorin and lumican. Many studies have found that both these proteoglycans are involved in the formation of fibrils and the regulation of the size of fibrils (Kangas, Edelhauser et al. 1990) and lumican has a bigger effect than decorin (Rada, Thoft et al. 1991). The corneal stroma has an inherent tendency to absorb water due to the proteoglycans' water-binding capacity, which is approximately 3.5 g H_20/g dry weight (Kaufman, 2003). Proteoglycans attract water into the cornea, whereas the endothelium pumps out the extra water from the stroma. There is a balance between these two activities, regulating the normal water content of the cornea. More detailed information about the water pump will be discussed in the section on endothelium.

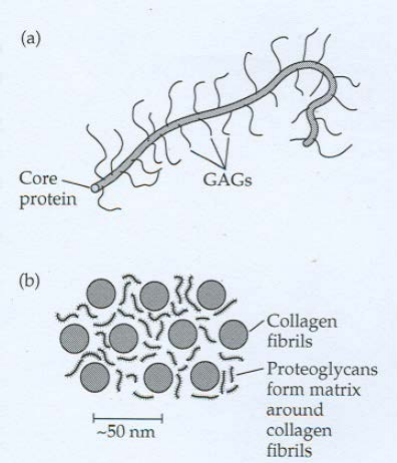

Figure 11: a) A cartoon diagram of a proteoglycan. It has a long core protein, with several GAGs extended to outside. b) Proteoglycans distribute between the collagen fibrils, forming a gel-like matrix to maintain the regular spacing (Oyster 1999)

There are small numbers of fibroblasts in the extracellular matrix (ECM) that form a communicating network through their connected cellular processes. The ECM of the stroma is produced and maintained by fibroblasts, which are called a keratocytes when found in the corneal stroma. Although these cells make up only 2-5% of the volume of the stroma (Oyster, 1999), they are critical for ongoing corneal functions (Nishida, Yasumoto et al. 1988). An individual keratocyte has a large, flattened cell body from which numerous slender processes extend (as Figure 12). When cultured, the fibroblasts grow until their processes touch other processes, forming an irregular meshwork of cells and processes (Stopak and Harris 1982). Where processes from neighboring cells touch, the cells may form gap junctions, so they can communicate with each other and coordinate their activities. During fetal development, the content of keratocytes is high

relative to that of collagen. Keratocytes produce both collagen fibrils and other extracellular matrix during development. Then, in a mature cornea, the volume of keratocytes drops down to about 2-3% of the total volume, and they play a role on maintaining regular fibril organization, as well as corneal repair after trauma.

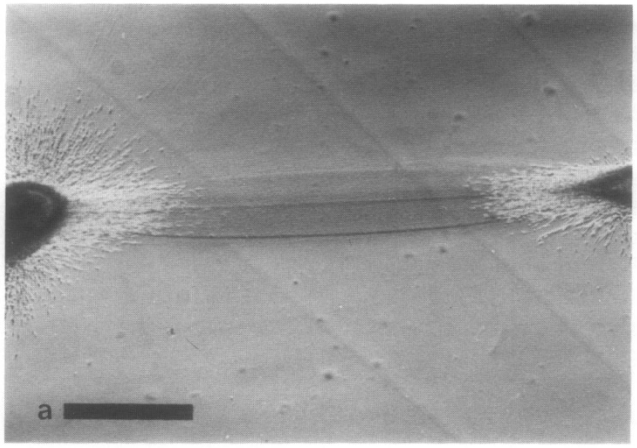

Figure 12: Two cultured fibroblasts extend their processes and connect with each other (Stopak and Harris 1982)

1.4 Descemet's membrane and Dua's Layer

Descemet's membrane is a thin layer, located between the endothelium and stroma, which thickens with aging. Its function is not very clear, but it is thought to serve as a barrier to corneal perforation (Eisenstein, Sorgente et al. 1973). In a young adult, the thickness of the Descemet's membrane is about 3 to 4 μm, and it grows throughout life, becoming approximately 10 to 15 μm thick. It is secreted by endothelial cells (Oyster, 1999) and is highly elastic, due to its components: type IV collagen, laminin, and

fibronectin (Kaufman, 2003). Even though Descemet's membrane is located between endothelium and stroma, it doesn't affect ion and water exchange (Kaufman, 2003). A recent study identified a novel layer, Dua's layer, which is located between the stroma and Descemet's membrane. This layer is strong and acellular, which is impervious to air and which may impact corneal biomechanics and pathology.

1.5 Endothelium

The endothelium is formed by a single layer of cells, which cover the entire posterior surface of the cornea (as Figure 3). It directly contacts the aqueous humor in the anterior chamber, and protects the corneal stroma from imbibing water.

One important function of the endothelium is to prevent the aqueous flowing into the stroma. Even though the thickness of an endothelium cell is only about 5 μm, the connecting surface between cells is highly interdigitated (Figure 13, right side), leading to an increased surface area (approximately 10 times greater). This makes it difficult for water to go between cells. In addition, cells are connected by tight junctions at the posterior part of the endothelial layer. These tight junctions have spot, instead of circular bonding, as is found in the epithelium.

Therefore, the endothelium cell layer resists water uptake, but cannot totally block it. In order to regulate the water content properly, ion pumps are found within the endothelium. They keep pumping the ions and water out of the cornea, making the endothelium a metabolic barrier. The importance of these activities is seen when the

endothelial water pumps are inhibited. In this case, water goes into the cornea, accumulating between the fibrils, leading to a loss of transparency. In summary, even though the endothelium can be thought of as a leaky membrane, it compensates for the leakage by its active water pumping, balance between inflow and outflow (Figure 14).

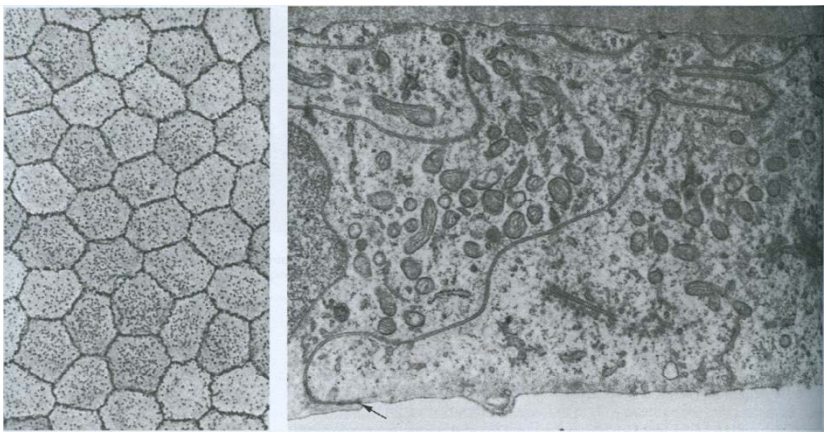

Figure 13: Diagram of endothelium under electro-microscope. The left diagram is the top view of the endothelium. The normal endothelium is six-sided shape with regular arrangement. The right diagram is the cross section view of the endothelium. Notice that there is only single cell layer, and the interface between endothelium cells are interdigitated. In addition, there are many mitochondria within the cell matrix. (Oyster 1999)

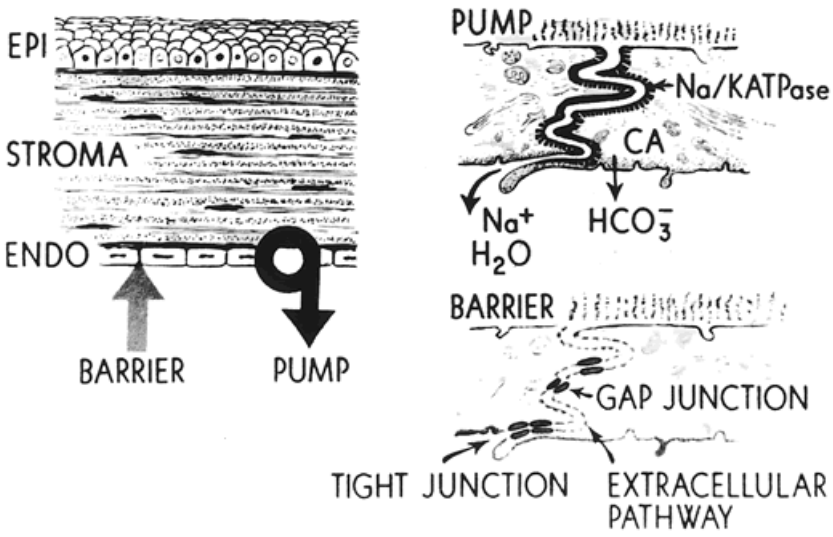

Figure 14: Diagram of the metabolic activities in the endothelial layer. The left figure shows the location and general function of the endothelium. The upper right image shows the pump location (intercellular space and posterior surface) and the simplified ions movements. The bottom right shows the junctions found within the endothelium layer (Waring, Bourne et al. 1982)

The density and the shape of the endothelium are changed by several factors. Normally, the density of the endothelial cells decreases with age. In a newborn baby, the cell density can be more than 5500 cells/mm, and it keeps dropping until it reaches 2500 to 3000 cells/mm in a mature person. However, the level is still far above the minimal functional level of 400 to 700 cells/mm (Bourne and Kaufman 1976). In aging, there are two main changes in the endothelial layer: one is a decrease in the number of cells and the other is a change in the shape of the cells (Yee, Matsuda et al. 1985). In a normal person, about 70% to 80% of the total cells are a regular hexagonal shape due to their

packing together to form a layer (Figure 13, left). However, under certain diseases or conditions, such as wearing contact lens (Holden, Sweeney et al. 1985), LASIK surgery (Collins, Carr et al. 2001), cataract surgery (Iradier, Fernandez et al. 2000) or having diabetes (Schwartzman, Balazy et al. 1987), the density of the endothelium decreased while the irregularity of individual cell shapes increased. This may due to stress, intraocular tension or abnormal components within the aqueous humor. Since endothelial cells cannot regenerate, the cells adjust to decreased density by enlarging and changing their shape to fill in the gaps left by dead cells, ending up with an irregular shape.

2. Tear Film

The tear film is the most anterior part the eye, covering the corneal surface. Traditionally, people believed that the tear film contained three distinct separated layers from posterior to anterior: mucus, aqueous and lipid layers (Figure 15). However, more a recent model (Figure 16) shows mixing of the aqueous and mucus layers, so that the aqueous layer becomes a mucin gel with a gradient of decreasing mucin concentration towards the lipid layer.

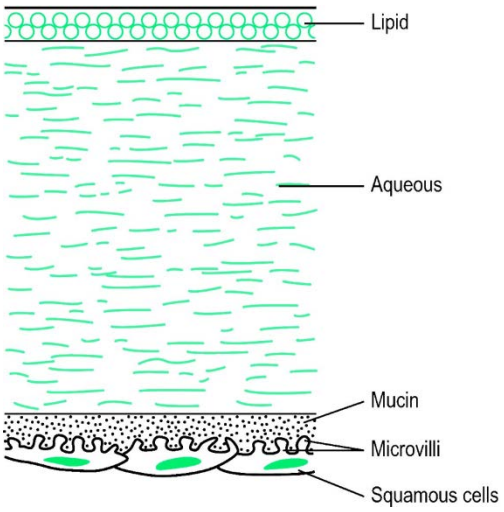

Figure 15: A traditional model of tear film structure. Notice that the three layers are separated sharply. Image from internet (http://medical-dictionary.thefreedictionary.com/tear+film)

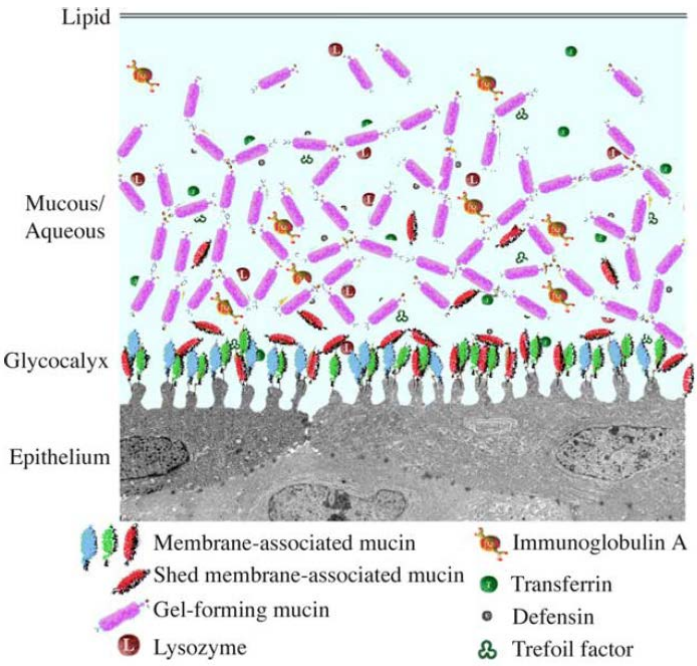

Figure 16: A new model of tear film structures. The concentration of mucin is gradually decreased moving toward the aqueous layer. The lipid layer is the anterior surface of the tear film. These three layers are not strictly separated. (Gipson 2004)

2.1 Mucin Layer

The mucin layer covers the corneal epithelium and contains different types of mucin molecules. The structure of the mucin molecule includes the heavily glycosylated glycoproteins and different numbers of amino acids in tandem repeats. Two basic categories of mucins have been identified: soluble mucins which are mainly secreted into the tear film by goblet cells in the conjunctival epithelium, and membrane-associated bound mucins which are secreted by the stratified squamous cells of the corneal epithelium. A small volume of soluble mucins are produced by the lacrimal glands. Different mucin molecules have different structures and distributions and the details will be discussed below.(Gipson and Argueso 2003; Gipson 2004)

2.1.1 Membrane-associated mucins

There are different types of membrane-associated mucins, and their structures are similar. Three types of membrane-associated mucins have been identified: MUC1, MUC4 and MUC16.Their structure involves a short cytoplasmic tail and a large extended extracellular domain. The hydrophobic short tail inserts itself into the epithelium, anchoring the mucin to the surface. The mucin's long extended hydrophilic part has many

repeating amino acids, which bond with serines and threonines in the tear film (Figure 17). Some mucins, such as MUC4, have epithelial growth factors between the tail region and the tandem repeats, which may contribute to the regulation of epithelial growth. Normally, membrane-associated mucins are associated with the epithelium. However, some mucins can move or float in the tear film. This type of mucin may be produced by the shedding of an epithelial cell and don't have the short anchoring part.

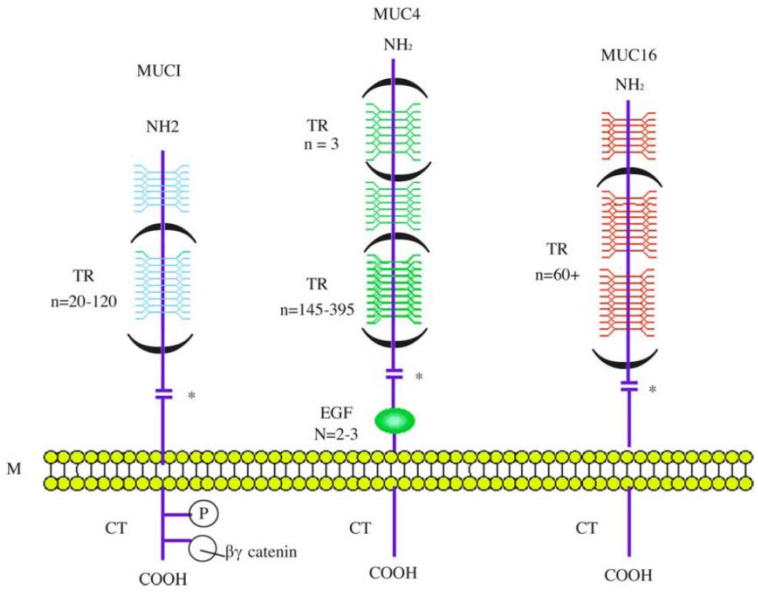

Figure 17: Diagram of of the cellular structures of the three types of membrane associated mucins. Abbreviation: M, epithelium membrane; TR, tandem repeats; CT, tail region; P, phosphorylated; EGF, epithelium growth factor. (Gipson 2004)

Membrane-associated mucins are mainly distributed over the epithelium, and are critical to the ocular surface health. As mentioned before, there are microvilli on the apical surface of the epithelial cells, increasing the interface between the tear film and

cornea. These three mucins are mainly distributed at the top of the microvilli and within the squamous cell layers (Figure 18). It is not clear whether each mucin has a specific function to the ocular surface. Some studies suggest that MUC16 on the ocular surface forms a barrier to pathogens (Argueso, Spurr-Michaud et al. 2003). All the membrane-associated mucins hold the water to the ocular surface by their hydrophilic properties, keeping the liquid spread over the surface. At the same time, floating mucins having the same electric charge produce a repelling force against each other during the eyelid movements. Therefore, there is less friction during the blink. The membrane associated mucins may contribute to surface wettability, protecting the ocular surface from bacteria (Gipson 2004).

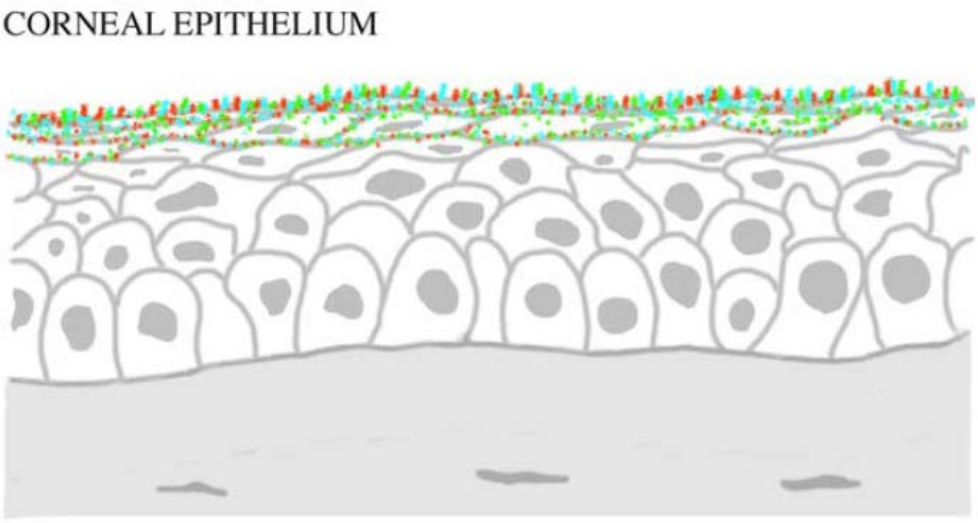

Figure 18: Diagram of the distribution of the membrane-associated mucins in the corneal epithelium. Different colors represent different types of mucins. (Gipson 2004)

2.1.2 Soluble Mucin

Several soluble mucins have been found, and they are mainly stored in the conjunctiva. Two types of soluble mucins have been identified: small soluble mucins, MUC7 and MUC9, and large gel-forming mucins MUCs 2, 5AC, 5B and 6. The MUC 5AC has been identified in the conjunctival epithelium. They are stored within vesicles in goblet cells (Figure 19). When the membrane of the vesicles melt into the conjunctival membrane, the MUC 5AC are released into the tear film. The goblet cells are found in the middle and superficial layers of the conjunctival epithelium. They are in high density in certain areas of conjunctiva, such as the plica semilunaris, and in the nasal side of the palpebral and forniceal regions. The goblet cells are fewer in areas of the conjunctiva close to the cornea.

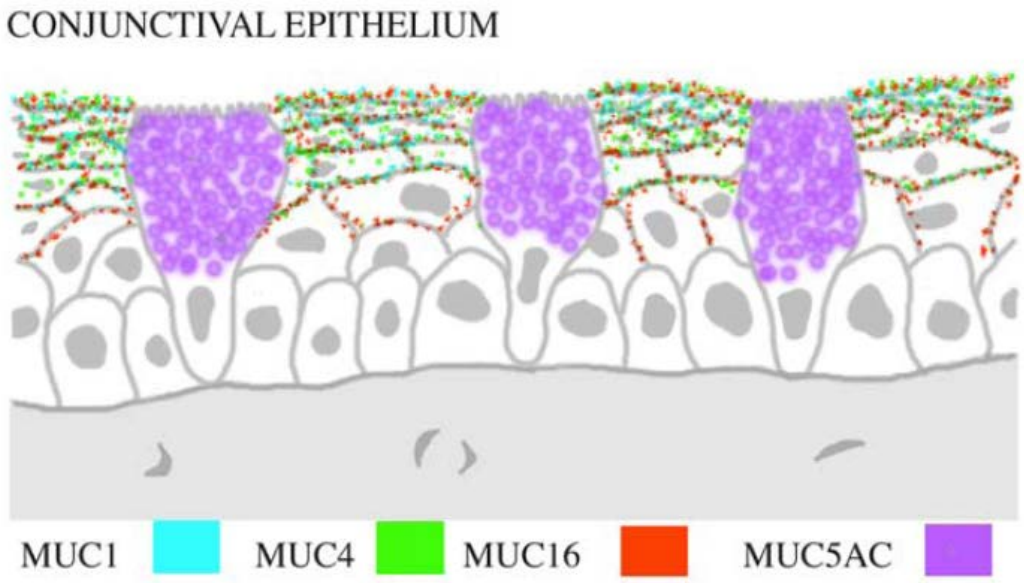

Figure 19: Diagram of the distribution of soluble mucins in the conjunctival epithelium. (Gipson 2004)

The distribution of the soluble mucins is variable, but it combines with the membrane-associated mucin to maintain ocular surface health. It is hard to study the soluble mucin distribution due to its solubility and the small volume of the tear film. The newer tear film model suggests that there is no sharp cutoff between mucin and aqueous layers, since the soluble mucin can move around. Because of the movement of the soluble mucins, the thickness of the mucin layer depends on the eyelid position. " As the eyelids move toward closure, the mucin layer may increase in depth and be moved toward the nasal puncta for elimination" (Gipson 2004).

In general, the membrane-associated mucins are located at the apical area of the corneal epithelium. They stay there by one side holding the aqueous to the surface; the other side anchoring the epithelium cell, and forming a barrier to the outside pathogens. The soluble and gel-forming mucins float on top of the membrane-associated mucins. They can move around, getting rid of the debris in the tear film and drain out through the nasolacrimal puncta. The membrane-associated, soluble and gel-forming mucins are all negatively charged, so there is repulsion between them, preventing surface adherence during the blink (Gipson 2004).

2.2 Aqueous Layer

The aqueous layer is the middle part of the tear film. Ninety percent of its content is water and the rest includes electrolytes, proteins, growth factors, immunoglobulins, cytokines, vitamins, and hormones. The aqueous layer is mainly secreted by the lacrimal

gland. Some other secretory issues, such as Krause, Moll, and Wolfring glands, also produce some components in the aqueous layer.(Levin and Kaufman 2011) Conjunctiva also plays a role in tear secretion (discuss later).(Dartt 1994)

Many proteins have been identified within the aqueous layer, and they play a big role in maintaining surface health. Major proteins include lysozyme, lactoferrin, secretory immunoglobulin A, serum albumin, lipocalin and lipophilin. Some of them have antimicrobial effects. For example, the lysozyme is an antimicrobial enzyme. It damages the bacterial cell walls to kill the pathogens. Lactoferrin is a metal binding protein. It combines with metal ions, critical for bacterial growth, thus preventing the spread of bacteria. Lactoferrin also can enhance the antibody activities to fight against certain microorganisms. Some of the proteins are growth factors, such as the epidermal growth factor and the transforming growth factor. They are activated for the wound healing process. In the dry eye diseases, some of these proteins have decreased in concentration, making the surface more fragile to pathogens (Kaufman, 2003).

Another important component in the tear film is ions, including sodium, potassium, magnesium, calcium, chloride, bicarbonate, and phosphate ions. One function of these ions, especially for the bicarbonate ions, is to maintain the tear film pH buffering ability. Since the pH is critical for enzymatic activity in cellular processes, a stable pH level is important in maintaining the corneal epithelial function. Studies have found that the pH of the tear film is relatively stable throughout the waking day, although slight fluctuations might be observed (Carney and Hill 1979). Normally, the pH of the tear is 7.4- 7.6, and the tears have a significant, but limited buffering capacity (Longwell, Birss

et al. 1976). If an acid is dropped (pH= 5.5) into the tear film, the pH drops to 6.0-6.5 immediately after the drop and then rapidly recalibrates to a pH of 7 in about 1 minute (Foulks 2007). However, if the pH of the solution is lower than 4.6 or higher than 9.0, it will not adjust as quickly and often creates discomfort after applying (Duke-Elder, 1962).

Another important function of ions in the tear film is to contribute to its osmolarity, which depends on the balance between tear secretion and drainage, and ocular surface evaporation. The 2007 Dry Eye Work Shop defined hyperosmolarity is a core mechanism for dry eye disease, along with tear instability.(2007) Increasing the surface evaporation or decreasing the tear secretion both lead to tear film hyperosmolarity. The tear breakup and thinning may lead to corneal surface exposed to the air or tear film hyperosmolarity, stimulating both epithelium and underlie receptors related to inflammation (Luo, Li et al. 2005; Luo, Li et al. 2007; Chen, Tong et al. 2008; Liu, Begley et al. 2009) and pain sensation.(Begley, Simpson et al. 2013) Long-term hyperosmolarity would also exacerbate the dry eye condition that promotes the cell apoptosis in conjunctival epithelium, decreasing the number of mucin-secretion goblet cells. (Baudouin, Aragona et al. 2013)

It is very difficult to measure the osmolarity of the tear film over the surface directly, due to the low volume of the tear film and lacrimal reflex. The normal tear osmolarity, measured from the lower meniscus, is 300-310 mOsM/kg (Holly 1973), and the cutoff for dry eye is 316 mOsM/kg (Gasymov, Abduragimov et al. 1999). However, the two osmolarity values from two groups were highly overlapped.(Tomlinson, Khanal et al. 2006) One possible explanation for this overlap was that above measurement might

not fully reflect the true osmolarity on the surface. The tear sample might be contaminated a lot by the lacrimation during the tear collection. (Mishima, Gasset et al. 1966; Liu, Begley et al. 2009)

Several mathematic models have been developed to understand the osmolarity change under different evaporation rate and tear volume.(Gaffney, Tiffany et al. 2010; Braun, Gewecke et al. 2014) Previous study in our lab had also found that the osmolarity within tear breakup might reach up to 800 mOsM/kg, which was well above the level for triggering inflammation and other cellular pathways. (Liu, Begley et al. 2009) Similar result was found in the mathematical model that a significant increase osmolarity could occur during the tear film thinning.(Braun, Gewecke et al. 2014) In addition, the tear meniscus osmolarity was lower than that on the ocular surface in both normal and dry eye subjects, but the difference was much higher in dry eye group, suggesting a high evaporation rate and low tear volume on dry eye patients. (Gaffney, Tiffany et al. 2010)

2.3 Lipid Layer

Chemical analysis of the tear film lipid layer (Figure 20) shows that there are polar and non-polar lipids (McCulley and Shine 1997) and lipid-binding proteins (Tiffany and Nagyova 2002). The non-polar lipid layer is the most anterior and comprises 60-70% of the lipid layer. It mainly contains wax, cholesterol esters, and triglycerides, which act to retard evaporation of the tear film, form a barrier to the outside environment and provide a smooth optical interface for the visual system. The polar lipid layer, containing

phospholipids and glycolipids, works as transitional region between the non-polar lipid layer and aqueous-mucin layer (Bron, Tiffany et al. 2004). The phospholipids are very strong surfactants in that they provide a surface to which the non-polar layer can attach (Butovich, Millar et al. 2008). Lipid-binding protein, lipocalin, is also found within this layer. Some studies suggested that it might play a role in decreasing the surface tension, thus stabilizing the tear film over the surface (Nagyova and Tiffany 1999). In addition, this protein may combine with other proteins, such as lactoferrin or secretory IgA, to contribute to normal tear viscosity (Pandit, Nagyova et al. 1999). In summary, the two sub-layers work together to decrease the surface tension, decrease the tear film evaporation, form a barrier to the outside environment, and provide a smooth optical interface.

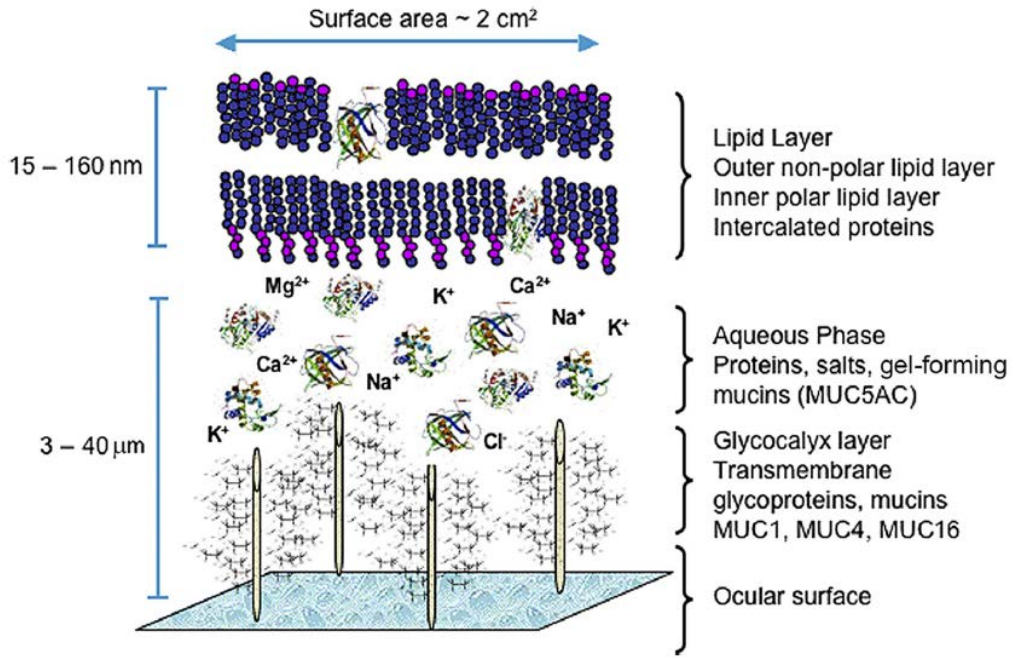

Figure 20: Diagram of a hypothetical model of the tear film with lipid and mucin distribution (Nichols, Foulks et al. 2011). The lipid and mucin layers are magnified to show the components clearly.

The kinetics of the lipids, including secretion, delivery, spread over the ocular surface and excretion, has been studied extensively. Lipids are mainly produced by the Meibomian glands, which are located within the tarsal plates. There are about 30-40 Meibomian glands in the upper lid, and 20-30 in the lower lid. Each gland consists of a bunch of grape-like acinar gland clusters, connecting to a long duct (Bron, Tiffany et al. 2004). The opening of the duct is on the skin of the lid margins, which are just anterior to the mucocutaneous junction. In this case, the Meibomian lipids are secreted within the gland clusters, move through the duct and are finally delivered to lipid reservoirs at the lid margins. “It has been assumed that meibomian oil is spread from the marginal reservoirs onto the tear film in the up-phase of each blink, after mixing with the lipid of the marginal reservoirs (Bron and Tiffany 2004).” That is to say, during the down phase of a blink, the lipid layer is compressed onto the lid margin. Since the lipid has a low melting temperature, close to the corneal, the lipid at the lid margin melts and mixes with the compressed lipid layer at the end of the down phase. Some lipids in the reservoirs attach and mix into the compressed lipid layer. During the up phase, the lipid layer is re-formed by mixing older and newly secreted lipid constituents. Immediately after the blink, the lipids move rapidly upward and then slow until finally they are entirely stationary, a process which takes about 1 second (Owens and Phillips 2001). The

subsequent flow of the lipid components out of the eye remains somewhat unclear. It has been suggested that part of the lipid flows out into the skin or eye lashes (Holly 1973), some may diffuse into the aqueous layer (Glasgow, Marshall et al. 1999) and some may drain into the nasolacrimal puncta (Himebaugh, Wright et al. 2003). After the initial upward movement, the lipid layer generally remains stationary within the interblink interval (Bron, Tiffany et al. 2004).

The Meibomian lipid level is variable and is regulated by different factors. Some studies suggest that the Meibomian lipid secretion is a continuous process day and night (Linton, Curnow et al. 1961), but that the eyelid movement during a blink helps this process. Studies show that the level of lipid at the lid margins is at the highest within one hour of waking and then decreases later in the morning, maintaining this level the rest of the day (Chew, Jansweijer et al. 1993). This result is correlated with the finding that the tear film thickness is higher in the morning, and lower in the afternoon (Franck 1991). This suggests that lipid secretion might be continuous during the night, storing a certain amount of oil in duct and lid margins. When the eye opens and blinking begins, the relatively high level of lipid collected overnight provides a thick lipid layer over the ocular surface, decreasing tear film evaporation (Bron, Tiffany et al. 2004). This hypothesis is also supported by observing lipid accumulation when blinking is suppressed by using anesthetics (Bron, Tiffany et al. 2004). In addition, forced, volunatary blinking produces a thicker lipid layer, suggesting that squeezing the eye increases lipid secretion (Korb, Baron et al. 1994).

Researchers also found that lipid levels at the lid margin increased with aging and the levels were higher in men than in women after puberty and before sixty (Chew, Jansweijer et al. 1993). Both androgen and estrogen receptors are found in the Meibomian glands (Bron, Tiffany et al. 2004). Lipid composition is changed with complete androgen insensitivity disease (Sullivan, Evans et al. 2002). Both lipid secretion and excretion decrease with aging. Since the amounts of lipid over the ocular surface and over the lid margin reservoir are 9 and 300 μg respectively, there is enough lipid stored at the lid margin to replenish the lipids over the ocular surface.

The evaporation of the tear film is decreased significantly with an abnormally thin lipid layer. A normal tear film lipid layer can reduce evaporation by about 90-95 percent (Tsubota and Yamada 1992), while the water loss increases by 10-20 times without the lipid layer in the rabbit (Mathers and Lane 1998). Studies have found correlations between the evaporation and the lipid layer thickness (Tiffany 1985). A change in the lipid composition has been reported in dry eye patients (Shine and McCulley 1998), as well as the thinner thickness of the lipid layer and an unevenly distributed lipid layer (Craig and Tomlinson 1997). However, it is not fully understood how the abnormal lipid layer interrupts tear film stability and how altered lipid composition may influence water loss.

3. Tear Film Thinning and Breakup

The stability of the tear film is usually measured by the tear breakup time (TBUT). Since the tear film is transparent, sodium fluorescein (a yellow fluorescent dye) is used clinically to show the tear film under cobalt blue light illumination. Fluorescein is instilled into the eye and it is spread quickly within a couple of full blinks. Subjects are asked to open their eyes as long as possible. TBUT is measured from when the subject fully opens the eye to the time in which the first dark spot is observed by the clinician (Fuchsluger, Steuhl et al. 2005). Cutoffs of 8 or 10 seconds are usual to define dry eye, and below that should be considered abnormal (Cho and Brown 1993; Lemp 1995; Avisar, Creter et al. 1997; Johnson, Murphy et al. 2008).

Several tear film breakup (TBU) patterns have been described in previous studies. A common pattern for TBU is the so-called “black line” (Donate, Benitez del Castillo et al. 2002). After applying fluorescein, two dark lines adjacent to the upper and lower meniscus can be visualized. Miller suggests that the black line is due to the liquid drawn from the ocular surface to the meniscus, resulting in the local thinning near the eyelids. The drawing force is derived from the capillary suction in the meniscus during the blink upward phase, creating a pressure gradient between the meniscus and the ocular surface (Otto and Roth 1996). Thus, this theory is often called the “thirsty meniscus” theory for TBU (Figure 21).(Miller, Polse et al. 2002) A similar black line can be found with partial blinks, but the mechanism is not clear (Dinic, Stojimirovic et al. 1977). Once the black line forms, TBU often occurs along its length. Previous study in our lab showed that TBU occurs repeatedly originating from the “black line”, suggesting that the surface structure might play a role in tear film instability. In addition, dry eye patients have higher TBU growth rate and more TBU in the central cornea compared to normal subjects, suggesting

that abnormal tear film and/or ocular surface structure on dry eye patients (Liu, Begley et al. 2006).

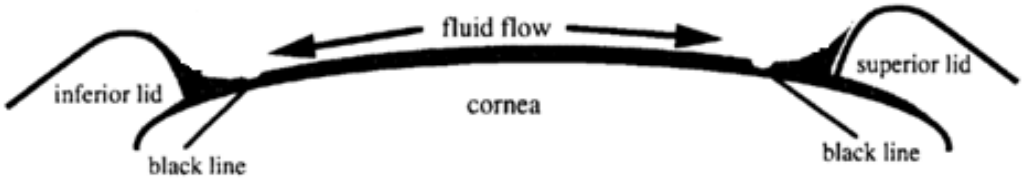

Figure 21: Diagram of the mechanism of the black line formation by pressure gradient (Rolando and Valente 2007)

Other types of TBU can occur in any pattern or at any location and are very common in dry eye patients. The patterns of the TBU have been described as “spots, stripes, or irregular pools” (Rosenfield 2011) . Studies suggest that these types of breakup might be related to sharp dry eye discomfort (Efron, Morgan et al. 2003; Liu, Begley et al. 2006). However, the mechanism of TBU and what exactly happens within the TBU area are not fully understood.

Several other models have been described to account for TBU, but none can fully explain all tear breakup conditions. Holly suggests a lipid contaminant model for TBU (Holly and Lemp 1977). In this model (Figure 22), some of the lipid molecules at the lipid-air interface diffuse or migrate into the aqueous layer, moving to the aqueous-mucin interface. Since lipids are hydrophobic, the attachment formed between lipids and mucin repels the aqueous from the adherence region, creating a hydrophobic area of TBU. Since the solubility of lipids is rather low, it is difficult for lipid diffusion to occur in the

aqueous layer in a short time. In addition, some studies show that the presence of the lipid layer is not necessary for tear film rupture. TBU is observed even in the event of complete destruction of meibomian gland openings. (Bron, Yokoi et al. 2011)

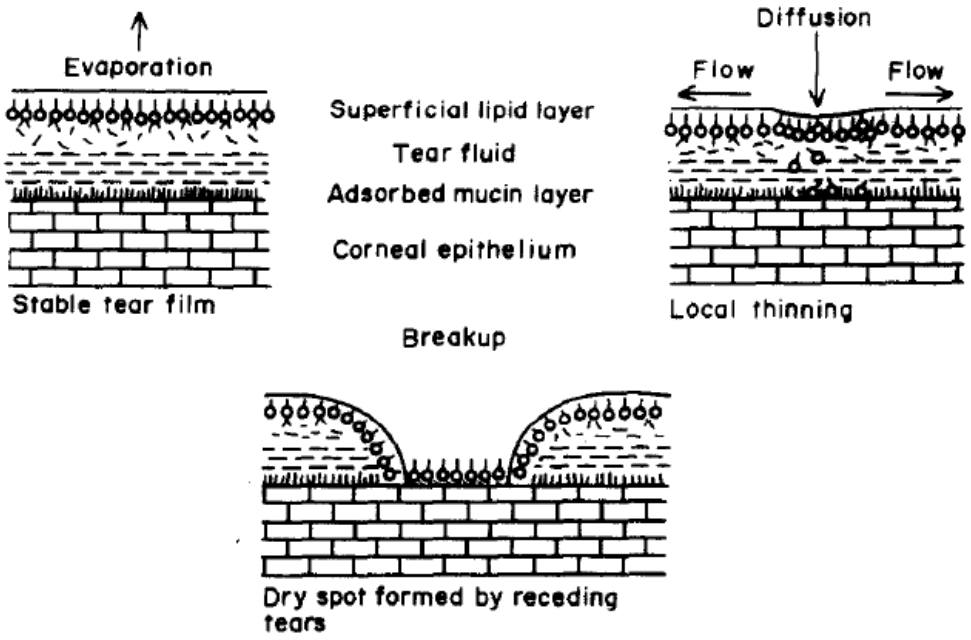

Figure 22: Diagram of Diagram of the mechanism of the tear film break up by lipid interruption (Holly and Lemp 1977)

Another mechanism for TBU is proposed by Sharma and Ruckenstein (Figure 23), who suggest that tear film rupture is due to the uneven distribution of the mucin layer (Bron, Yokoi et al. 2011). In this model, a thin mucin layer is formed after a blink. The van der Waals dispersion forces act on this thin mucin layer, and rupture the mucin film. Since the mucin is a hydrophilic interface between the hydrophobic epithelium and aqueous layer, the rupture of the mucin layer exposes the epithelium to the aqueous layer,

resulting in TBU. However, more recently, attached membrane-associated mucins have been shown to cover the epithelial surface (Gipson and Argueso 2003). It is likely that van der Waals dispersion forces are not large enough to disrupt the tear film over this bound mucin layer. Thus, this model does not appear to be physiologically probable.

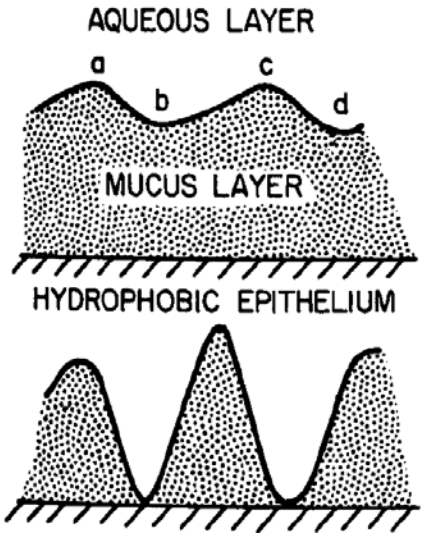

Figure 23: Diagram of the mechanism of the tear film breakup by uneven mucin layer (Bron, Yokoi et al. 2011)

McDonald and Brubaker also proposed another tear breakup model where the tear film flows over the ocular surface into the upper and lower menisci after a blink, theoretically due to different hydrostatic pressures. However, this model does not explain how tear breakup happens at different locations in irregular patterns. Recently, King-Smith proposed that tear film breakup is mainly caused by evaporation (King-Smith, Nichols et al. 2008). In this paper, he pointed out that there was no single model that could explain all the cases of tear breakup. The inward aqueous flow had the least

importance in tear breakup formation, in contrary, there was an outward aqueous from corneal epithelium to tear film due to the osmolarity gradient. In addition, the tangential flow may play an important role in certain tear breakup conditions, such as black line formation near the meniscus or after a partial blink. The evaporation probably is the main component for tear film thinning and breakup.

4. Conjunctiva

The conjunctiva is a continuous mucous membrane which makes up of three parts. The palpebral part lines the posterior surface of the eyelids; the bulbar part covers the most anterior surface of the globe, extending to the limbus; the forniceal part is the junction between palpebral and bulbar parts. The conjunctiva is composed of a stratified secretory epithelium and an underlying substantia propria. Several functions of conjunctiva are including: 1) allows the eyelids move over the ocular surface freely with minimal resistance; 2) produce the tear film aqueous and muscin (goblet cell); 3) fornix acts as a tear reservoir which contains half of the total tear liquid on the ocular surface.

5. Corneal Innervation

5.1 General information

The innervation of the cornea has been studied before. The cornea has the richest sensory nerve endings in the body. The cornea is innervated by the ophthalmic branch of

trigeminal nerve, through the anterior ciliary nerves (Oliveira-Soto and Efron 2001). All of the peripheral axons lose their myelin sheaths when they enter the middle third of the corneal stroma. They run forward and anteriorly, branching out extensively, toward the center of the cornea (Figure 24). The stromal nerves form a subepithelial nerve plexus at the interface between Bowman's layer and the anterior stroma. Then they go across the Bowman's layer, forming the subbasal epithelial nerve plexus. This nerve plexus, which runs parallel to the corneal surface and projects its nerve endings into the superficial layer, innervates the basal epithelial cell layer and terminates within the superficial epithelium (Figure 27). Even though some nerve endings are in the stroma, there is still an extremely high density of nerve endings (2500 terminals/ mm^2) in the superficial epithelial layer, explaining why the cornea is so very sensitive (Oyster, 1999) .

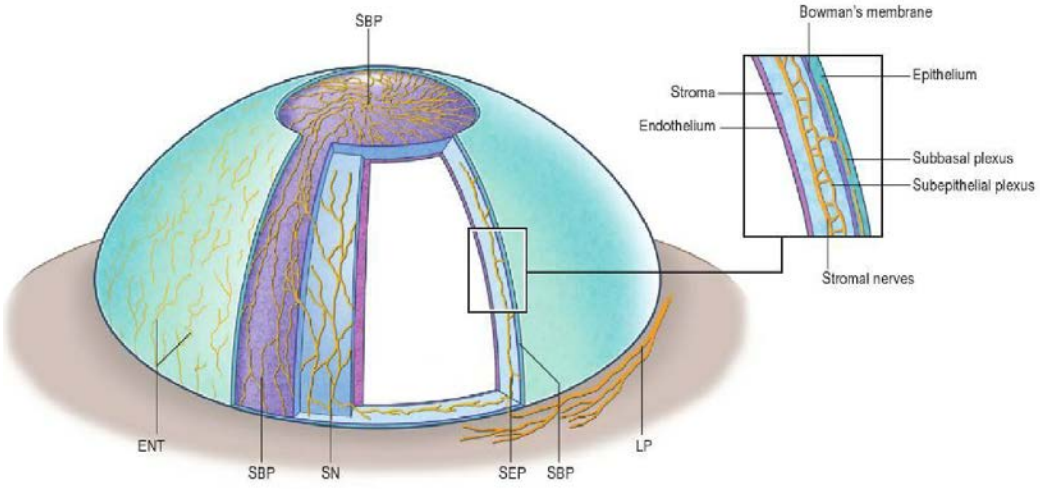

Figure 24: Diagram of the distribution of nerves in the cornea. (Levin and Kaufman 2011) ENT: intraepithelial nerve terminals, SBP: sub-basal plexus, SN: stromal nerve trunks, SEP: subepithelial plexus, LP: limbal plexus

The nerve fibers enter the cornea from the limbus and they branch and anastamose repeatedly to form a high density plexus in the cornea. At the point of entry, about 70-80% of nerve fiber lose their myelination (C fiber) whereas the rest fibers (A-δ fiber) still have a very thin myelination. Some nerves terminate in the stroma as free nerve endings, whereas some penetrate the Bowman's layer and enter the epithelium to form sub-basal plexus. As Figure 25 shown, the C fibers run upward across the whole layers of the epithelium and their pathway are almost perpendicular to the ocular surface, whereas the A-δ fiber mainly stay in the basal and wing layers and mainly run parallel to the ocular surface.

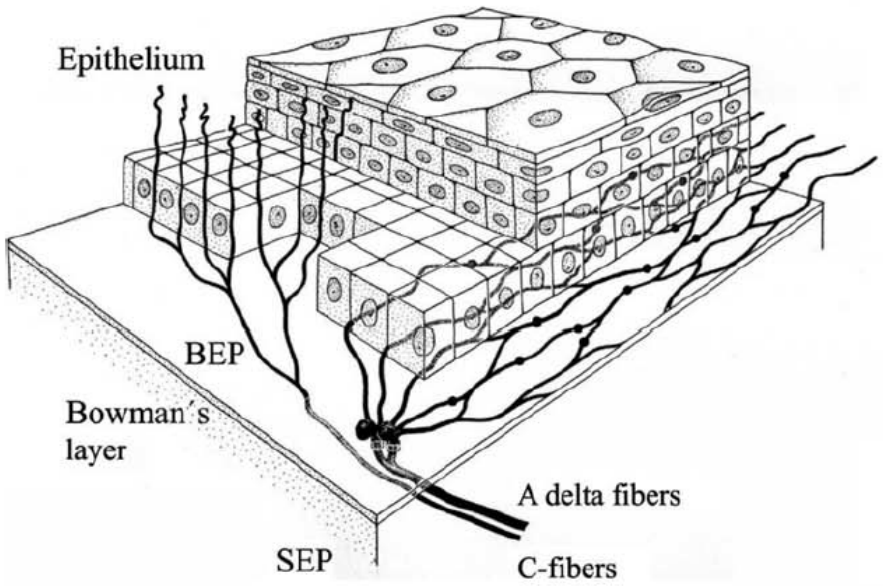

Figure 25: Three-dimensional drawing of the penetration and distribution of nerve fiber running into the sub-basal plexus. SEP: subepithelium plexus, BEP: basal epithelium plexus (sub-basal plexus) (Guthoff, Wienss et al. 2005)

5.2 Corneal Receptors

Corneal nerve fibers can be classified as A-delta or C types, depending on their diameters or whether have myelin sheath. (MacIver and Tanelian 1993; MacIver and Tanelian 1993; Belmonte, Garcia-Hirschfeld et al. 1997) A-delta fiber has a relative thick axon and a thin myelin sheath around, which makes the A-delta neuron conduct a signal much faster (velocity= 2~15m/s) than that of the C neuron which has a thinner axon without a myelin sheath (velocity< 2m/s). (Belmonte and Gallar 2011) This difference of conducting velocities between A-delta and C fibers results in the so-called "first and second pains" experienced with tissue injury. For example, a foreign body in the eye will result in an immediate sharp, intense "first pain", followed by a more prolonged dull "second pain". The immediate pain is transmitted by A-delta fibers, which create a large, but short, action potential. The second, dull pain is transmitted by C fibers, which create a relatively low but continual potential.(Kandel, Schwartz et al. 2000) There are mainly three types of sensory neurons, including chemical, mechanical and thermal neurons, innervating the ocular surface. (Levin and Kaufman 2011) Most of them belong to the general group of nociceptors, which will be activated by noxious or close-noxious stimuli related with injury and tissue damage. (Belmonte, Aracil et al. 2004).

The first type is called polymodal nociceptors, which comprise more than 70% of the total corneal receptors. They are activated by three types of stimuli: 1) near-noxious or noxious mechanical force; 2) heat and noxious cold, 3) external chemical irritation (CO2 and acid solution) and internal chemical mediators released from inflammation and

damaged tissue. (Belmonte and Gallar 2011) Most of the polymodal nociceptors are C fibers, and they are activated as long as the stimulus is maintained. Their firing rate is roughly proportional to the stimulus intensity. Therefore the polymodal nociceptors can encode the intensity and duration of the stimulus. (Belmonte, Aracil et al. 2004) Most of time, polymodal nociceptors remain inactivated after stimulation. However, when there is tissue damage, the polymodal nociceptors start to discharge continually and irregularly, leading to sensitization. The phenomenon of sensitization includes 1) decreased thresholds to three types of stimuli (mechanical, chemical and thermal stimuli); 2) increased responses to suprathreshold stimuli, and 3) development of an ongoing irregular spontaneous discharge without stimulus. Therefore, the neuron sensitization might contribute to hypersensitivity, allodynia and hyperalgesia, which are found with long-term dry eye patients. (Belmonte, Garcia-Hirschfeld et al. 1997; Belmonte, Acosta et al. 2004; Belmonte and Gallar 2011)

About 15% of the corneal neurons with A-delta type fibers are activated by mechanical stimuli close to the noxious level, which may damage normal tissues. (Belmonte and Gallar 2011) They encode the presence and intensity (in very limited range) of stimuli, but do not encode its duration. (Belmonte, Acosta et al. 2004; Belmonte, Aracil et al. 2004) Therefore, they are fast-adapted neurons. (Chen, Feng et al. 2010; Chen and Simpson 2011) The threshold of mechanical receptor is slightly higher than the polymodal receptors, but much lower than that in on the skin. However, the stimulus level might be enough to hurt the corneal surface. (Belmonte and Gallar 2011)

Another category of corneal neurons (10-15%) is the cold-sensitive receptor, named cold thermoreceptors. The thermoreceptors normally discharge spontaneously under normal corneal temperatures. They immediately increase their firing rate when corneal temperature drops, and then they stabilize to a higher firing rate proportional to the magnitude of the temperature drop. Therefore, the thermoreceptors can encode the presence, the amount of temperature drop and the final temperature on the ocular surface. Several studies have found the thermoreceptors can detect temperature drops as small as 0.5ºC. (Belmonte and Gallar 2011)

One main function of the thermoreceptor is to maintain the basal tear secretion by detecting small temperature fluctuations on the ocular surface. (Belmonte, Aracil et al. 2004; Hirata and Meng 2010) During the interblink interval, the temperature on the ocular surface drops about 0.3 ºC/s due to evaporation. (Fujishima, Toda et al. 1996) Since thermoreceptors are highly sensitive to temperature changes, they increase their discharge rate, sending signals to the superior salivary ganglion in the brainstem, which finally project onto the lacrimal gland for tear secretion. (detail information discussed later). Under normal conditions, basal tear secretion is thought to be maintained by the tonic activity of the cold thermoreceptors. (Belmonte and Gallar 2011) Interestingly, the amount of basal tear secretion was not increased with moderate cool stimulation, suggesting “the activity of cold thermoreceptors within the normal range of corneal temperatures (36-29°C) in healthy eyes appears to exert a nearly maximal stimulatory effect on tear secretion.” (Parra, Madrid et al. 2010; Belmonte and Gallar 2011)

When the thermal stimulus is higher than the normal range, above the noxious level, there was a significantly large tear flow onto the ocular surface, termed reflex tearing. Studies have shown that the reflex tearing occurred when a strong noxious stimulation was applied, suggesting that reflex tearing might be initiated by polymodal nociceptors activation. This reflex tear secretion might be independent from basal tear secretion, which was mainly modulated by cold thermoreceptors. (Belmonte, Aracil et al. 2004)

5.3 Corneal Sensitivity

5.3.1 Cochet-Bonnet Corneal Esthesiometer

The Cochet-Bonnet corneal esthesiometer is the most widely used clinical instrument for quantifying corneal mechanical sensitivity (Millodot 1984). It is composed of a thin flexible nylon thread, which is applied to the human eye with variable lengths to produce different forces. The corneal threshold is quantified by the length of the nylon filament.

Several factors contribute to normal variations in corneal sensitivity, as measured by Cochet-Bonnet. Corneal sensitivity is greater in the central area than that in the peripheral area (Millodot and Larson 1969). After the age of 50 years old, corneal sensitivity decreases significantly, down to half the normal value at 65 years old (Jalavisto, Orma et al. 1951; Boberg-Ans 1955; Draeger 1979). Iris color is also related with corneal sensitivity. People with blue eyes are more sensitive than those with brown

eyes (Millodot 1975). In addition, there is a diurnal variation, with sensitivity higher in the evening than in the morning (Millodot 1972). Finally, pregnancy, menstruation (Millodot 1977), low temperature (Millodot 1984) and radiation (Millodot and Earlam 1984) can decrease the sensitivity.

Wearing contact lens, surgery and disease can also influence corneal sensitivity. Several studies found that wearing contact lenses can decrease corneal sensitivity significantly (Boberg-Ans 1955; Knoll and Williams 1970) and takes time to recover after the lenses are removed (Millodot 1975). If the contact lenses are worn for long time, longer period is needed for recovery (Millodot 1984). The mechanisms of this abnormal sensitivity might be due to the metabolic impairment arising from low oxygen diffusion through the contact lenses or the mechanical stimulation arising from contact lens movement (Millodot 1984). In addition, some diseases and surgery, such as corneal ulcers and LASIK surgery alter corneal nerve structure, leading to abnormal corneal sensitivity.

However, there are some limitations of the Cochet-Bonnet esthesiometer. First, it is an invasive measurement that can induce corneal injury during the process. Secondly, applying a nylon thread to the corneal surface is a very complex stimulus. Besides the length, the thread's shape, bending angle, moisture level and contact location can all influence the detection of the stimulus and threshold measurement. Since the cornea is highly sensitive, stimuli with high precision and specification are needed to measure the sensation response thresholds.

5.3.2 Belmonte Gas Esthesiometer

In recent years, the gas esthesiometer was developed as an alternate tool for measuring corneal sensitivity. The gas esthesiometer does not touch the ocular surface, and allows a precise localization of stimuli and controls the air velocity, temperature and CO_2 content, putatively to measure mechanical, thermal and chemical corneal sensitivities, respectively.(Murphy, Patel et al. 1996; Belmonte, Acosta et al. 1999; Feng and Simpson 2003; Stapleton, Tan et al. 2004)

An air puff with adjustable air velocity is used to provide a mechanical stimulus and the thermal effect in this stimulus is avoided by warming the air to the body temperature. Thermal stimulus is provided by varying the air temperature and the mechanical component in this stimulus was controlled by setting the flow rate below subject's mechanical threshold. Chemical stimulus provided by the Belmonte gas esthesiometer composed of CO_2, which dissolves in tears decreasing the tear film pH. Corneal pH decreases, due to the formation of carbonic acid, although the exact pH change in the tear film has not been measured.(Belmonte, Acosta et al. 1999)

Although the stimulus from the Belmonte esthesiometer has been carefully controlled, the exact nature of the stimulus is still not well understood and it is possible that single stimulus contains different stimuli components. For instance, any air flow might induce evaporation, prompting cooling and hyperosmolarity on the surface. Thus, the mechanical stimulus, especially at high flow rates, may also contain thermal and chemical components. In addition, the buffering capacity of the tear film may alter the chemical stimulus induced by carbonic acid, making the stimulus less intense.

The Belmonte esthesiometer has been used to study the ocular surface sensitivity in a variety of clinical settings. For example, it is well known that contact lenses alter corneal sensitivity. Studies found that there was a positive correlation between the levels of corneal staining, a measure of surface damage, and conjunctival chemical sensitivity (Situ, Simpson et al. 2010). In addition, corneal sensitivity differs between symptomatic and asymptomatic subjects. Researchers found that the threshold for all three stimuli presented by the Belmonte esthesiometer were significantly higher in dry eye patients than in control subjects (Bourcier, Acosta et al. 2005). However, in another study, symptomatic subjects had lower thresholds than normal subjects (Situ, Simpson et al. 2008). These studies suggested that dry eye subjects not only had unstable tear film, but also abnormal corneal sensitivity. Futhermore, there was a mechanical and thermal adaptation to suprathreshold stimuli in the normal subjects, but not to chemical stimulus (Chen, Feng et al. 2010; Chen and Simpson 2011) suggesting the functional difference between corneal fibers (Aδ and C fibers),(MacIver and Tanelian 1993) underlie signal pathways and processing in the central nerve system.

Although the nature of the stimuli delivered by the Belmonte aesthesiometer are not clear, it provides a possibility of a controlled, localized, laboratory-based method to further study the effects of ocular surface stimulation on blinking, tear secretion and subjective sensation.

5.3.3 Ocular Surface Sensation

The mechanisms for causing ocular surface discomfort are not clear. Eye irritation is very common, especially for dry eye patients, (Doughty, Fonn et al. 1997; Schein, Munoz et al. 1997) contact lens wearers (Begley, Caffery et al. 2000; Begley, Chalmers et al. 2001) and after LASIK surgery. (Ambrosio, Tervo et al. 2008) Corneal pain sensations can be induced by activation of corneal nociceptors, especially the polymodal nociceptors. Since the polymodal nociceptors contain both A-delta and C types of axons, the pain produced is acute and sharp at the beginning (A-delta type), and the dull, long-lasting pain is felt afterward (C type).(Belmonte, Acosta et al. 2004; Belmonte, Aracil et al. 2004) Previous studies show that nerve fiber discharge increases rapidly when a stimulus is applied (Belmonte and Giraldez 1981). The neural firing rate increases, reaching a maximum value if tissue injury occurs. Removal of the noxious stimulus stops the discharge temporarily, but it reappears a few seconds afterwards as an irregular, low frequency firing pattern. This pain may be due to sensitization, which arises from the acutely injured tissues, leading to increasing spontaneous neuron excitability. Some studies also show that when the corneal nerves are cut, the injured area is first invaded by sprouts from adjacent healthy fibers, and as the affected nerve branch degenerated, the new generated fibers enter and form microneuromas (Beuerman and Rozsa 1984; Trabucchi, Brancato et al. 1994). This regeneration is always accompanied by abnormally functioning nerve endings. The newly-generated nerve fibers are created by over- or under-expressed genes, encoding dysfunctional membrane proteins and ion channels, etc. This distorts the normal corneal sensitivity, ending up with abnormal responses to external stimuli and irregular spontaneous activities. These abnormal nerve endings can

contribute to chronic pain and lower sensitivity when external stimuli are applied (Belmonte, Acosta et al. 2004).

In summary, corneal sensations probably depend on different combinations of activated nociceptors and other neurons. Ocular surface disease or surgery may injure the normal sensory system, and the irregular nociceptor activity may cause the resulting surface discomfort. Psychophysical studies showed that the intensity of the stimulus, the discharge rate of nerve fibers, the number of nerve fibers recruited, and the subjective intensity of the sensation are directly correlated.

5.3.4 Molecular Mechanisms of Sensory Information Transported underlie Neurons

One characteristic of sensory neurons is that they are able to respond to many different signals from the outside environment. These stimuli are converted into electrical or chemical signals that the brain can understand. Several transient receptor protein (TRP) channels have been identified in the cornea, reacting to various stimuli. (Stapleton, Marfurt et al. 2013)

TRPV1 channels are the main receptors for detecting chemical and heat stimuli at the noxious level. They are found on both A-δ and C neurons and integrate chemical and thermal stimuli together. (Fukuoka and Noguchi 2006) Chemical mediators released during inflammation might modulate the TRPV1 activities, leading to sensitizing on nociceptors.(Stapleton, Marfurt et al. 2013)

TRPA1 channels are expressed on C type nociceptors that also express TRPV1 channels. (Story, Peier et al. 2003) TRPA1 is activated by a range of diverse stimuli, including chemical mediators and molecules, mechanical force and cold stimulus. The chemical mediators, which might be released from inflammation or damaged tissue, act on TRPA1 channels triggering burning and prickling sensations. (Stucky, Dubin et al. 2009) Similarly, TRPA1 integrate diverse stimuli together to modulate the neuron activities. (Stapleton, Marfurt et al. 2013)

TRPM8 channels are mainly expressed on cold thermoreceptors and specifically respond to cold stimuli. (Belmonte and Gallar 2011) They are highly sensitive to small temperature drops (<2ºC) that a cooling stimulus will induce and gradually adapt to new neural discharge rates in a manner proportional to the final temperature. (Hirata and Meng 2010; Parra, Madrid et al. 2010) The transient increment of the nerve terminal impulses normally last 30 seconds (dynamic period) and then remain stable (static period). (Parra, Madrid et al. 2010) Some cold thermoreceptors only respond during the dynamic period and then revert back to their normal nerve terminal impulse levels.(Hirata and Meng 2010)

Acid-sensing ion channels and potassium channels also play a role in detecting different types of stimuli. The acid-sensing ion channels respond to several chemical mediators released from local inflammation. The potassium channels are involved in detecting thermal and mechanical stimuli. (Uchino, Nishiwaki et al. 2011)

Hyperosmolarity is a common chemical stimulus that is thought to occur in the dry eye condition. Recent studies have shown the effects of hyperosmolarity on the nerve

terminal impulse activities of cold and polymodal nociceptors. (Hirata and Meng 2010; Parra, Gonzalez-Gonzalez et al. 2014) Surprisingly, the small osmolarity increments, which could occur early in tear breakup or thinning, was mainly detected by cold thermoreceptors. There was a linear relationship between solution osmolarity and the increment of cold thermoreceptor firing rate, when the osmolarity ranged from 325 to 480 mOsM/kg. (Parra, Gonzalez-Gonzalez et al. 2014) Polymodel nociceptors were not activated until the osmolarity reached 600 mOsM/kg. Other studies showed the cold thermoreceptor response was saturated when the osmolarity reached around 1500 mOsM/kg. (Hirata and Meng 2010) Hyperosmolarity solutions significantly increased the firing rate of thermal receptors and increased nerve terminal impulses to the same thermal stimulus. These results showed that the activating the corneal receptors depends both on the stimulus type and its intensity.

6. Conjunctival Innervation

Less information is available for conjunctival innervation compared to the corneal sensory system. As mentioned before, the conjunctiva is separated into three parts: the palpebral, fornix and bulbar conjunctiva. Sensory innervation of the bulbar and palpebral conjunctiva is supplied by branches from ophthalmic nerve (one branch of trigeminal nerve).(Oyster 1999) Most conjunctival fibers are unmyelinated, except a few fibers have thin myelination. A few bulbar conjunctival fibers originate from large-diameter, heavily myelinated axons and terminate as Krause corpuscles.(Lawrenson and Ruskell 1991) These Krause corpuscles are found all over the bulbar conjunctiva and the highest density

occurs in a 1mm wide annular region outside the limbus. The function of the Krause corpuscle is still unclear, but it might be related to fast adaptation to tactile stimulation.(Stapleton, Marfurt et al. 2013)

The conjunctival sensitivity has been measured by both Cochet-Bonnet and air esthesiometers. From the studies using Cochet-Bonnet measurement, the tactile sensitivity is much lower in conjunctiva compared to that in the cornea.(Norn 1973) Similar result was found by using the air esthesiometer.(Vega, Simpson et al. 1999; Acosta, Alfaro et al. 2006) Due to the relative lower nerve fiber density in conjunctiva compared to cornea, the conjunctival sensitivity to pneumatic stimulus at room temperature is lower than that of the cornea, especially in symptomatic patients. Since the conjunctiva contributes to aqueous tear film and mucin secretion, this result suggests that the sensory system might be more impaired in the symptomatic group, which is critical in the dry eye condition.(Situ, Simpson et al. 2008) Regional differences in conjunctival sensitivity have also been reported. The limbal conjunctiva is more sensitive to mechanical stimulation compared to the palpebral and bulbar conjunctiva.(Lawrenson and Ruskell 1993)

7. Lacrimal gland

7.1 General information of lacrimal gland

The main lacrimal gland is an almond-shaped gland which is located in the superior temporal bony orbit. It is a tubuloacinar exocrine gland that secrets the major

portion of proteins, electric ions and water in the tear film. Different types of proteins are secreted by the lacrimal gland, including the anti-bacterial proteins, immunoglobulins, growth factors and extra. These proteins are important to maintain the ocular surface health under a variety of external environments.

The major cell type in the lacrimal gland is the acinar cell which consists 80% of the total cells. Acinar cells are pyramidal shaped and are linked together by the tight junctions at the lateral apical portion of the membrane (Figure 26) to form a ball in the gland portion and tube in the duct portion. The tight junctions are responsible for the polarization of the acinar cell, making sure the unidirectional secretion of aqueous, protein and electrolytes from cell plasma into the lumen. The receptors on the basolateral membrane can response to several neurotransmitters, such as acetylcholine and VIP, to initiate the secretion. The apical membrane is the site of granules fusion that delivers the proteins into the lumen.

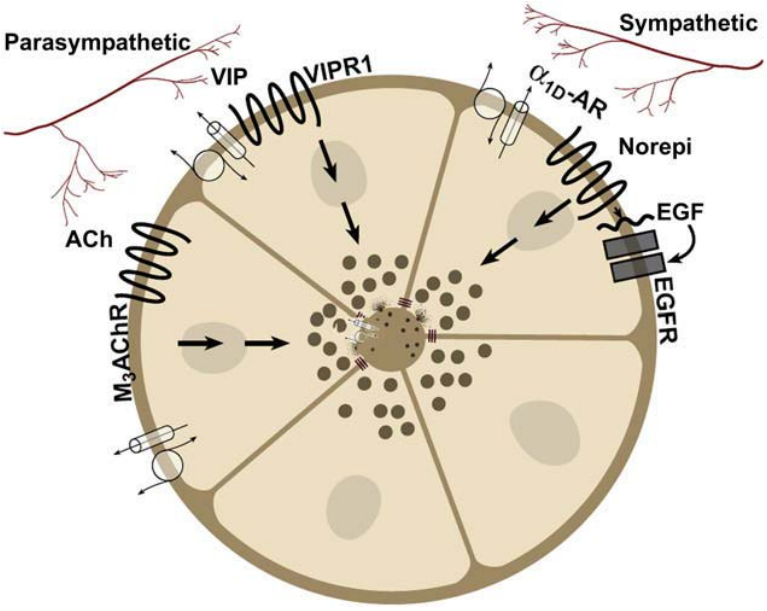

Figure 26: Schematic of the structure of the lacrimal gland (Dartt 2009)

The lacrimal gland ducts are lined with one to two layers of cuboidal duct cells that make up 10% of the total cells. Similar to acinar cells, duct cells are also linked on the apical side by tight junctions, creating polarized cells that contribute to the unidirectional secretion of the lacrimal gland fluid. One important function of the duct cells is to modify the primary lacrimal gland fluid from the acinar cells by secreting ions and water while it delivers tears to the ocular surface. Based on a previous study, the lacrimal gland duct cells secrete about 30% of the lacrimal gland fluid.(Mircheff, Lu et al. 1983; Dartt 2009)

The third cell type present in the lacrimal gland is the myoepithelial cells. These cells surround the basal side of both acinar and duct cells. In the guinea pig lacrimal gland, the myoepithelial cells have been observed to contract in response to the cholinergic agonist carbachol. However, their function in the tear production is unclear.

7.2 Neural stimulation of tear secretion

The corneal fibers travel through the long posterior ciliary nerve in the sclera and join the nasociliary nerve as the long sensory root. The nasociliary nerve travels within ophthalmic nerve and exits the orbit. The sensory nerve passes through the trigeminal ganglion and enters the trigeminal brainstem nuclear complex. The secondary ocular sensory neurons are mainly located in the intermediate zone between interpolaris and

caudalis (Vi/Vc) and in laminae I-II of the subnecleus caudalis/upper cervical spinal cord (Vc/ C1). The output of the sensory nerve starts at the superior salivatory neclei. From here, the efferent fibers go into the facial nerve, passing through the geniculate ganglion, and synapse on the pterygopalantine ganglion. This nerve, which contains parasympathetic fibers, goes through the maxillary nerve (one branch of the trigeminal nerve), zygomatic nerve (one branch of the maxillary nerve) and lacrimal nerve (one branch of the zygomatic nerve) to finally projects onto the lacrimal gland. (Figure 27) While the parasympathetic nerve fibers go through the pterygoid channel and project on the pterygopalantine ganglion, it forms a retrobulbar plexus of nerves that including the sympathetic fibers from plexus around carotid artery. Both sympathetic and parasympathetic outputs mediate the tear secretion.

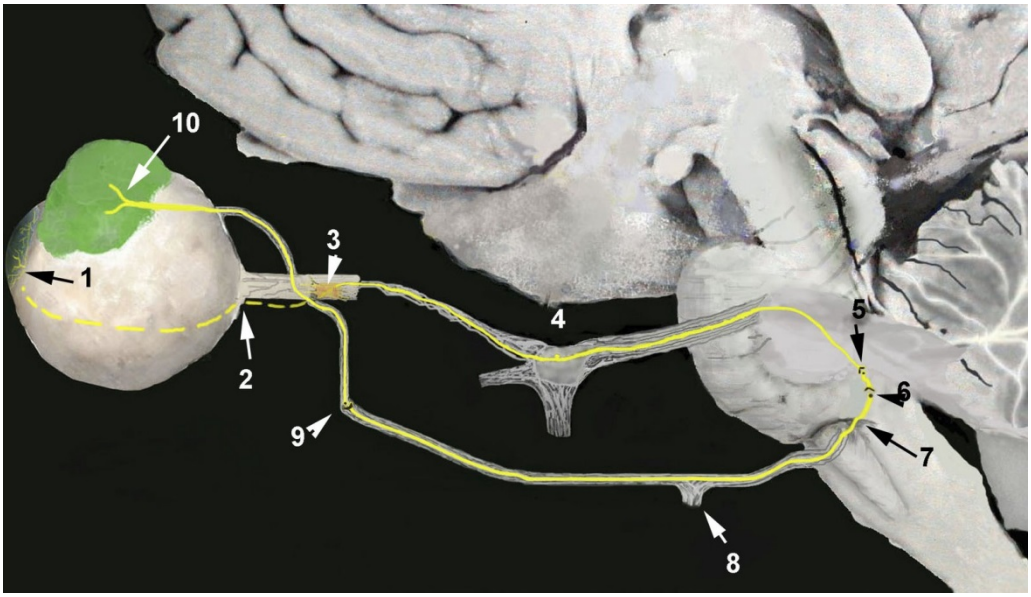

Figure 27: the lacrimal nerve pathway. Arrow 1: sensory fiber from cornea. Arrow 2: ciliary nerve. Arrow 3: ciliary ganglion. Arrow 4: trigeminal ganglion. Arrow 5:

trigeminal brainstem nuclear complex. Arrow 6: salivatory neclei. Arrow 7: facial nerve. Arrow 8: geniculate ganglion. Arrow 9: pterygopalantine ganglion

Activation of either parasympathetic or sympathetic nerves releases neurotransmitters that regulates lacrimal gland secretion of proteins, electrolytes and water. The major neurotransmitters that control secretion are the parasympathetic neurotransmitters, including acetylcholine (Ach) and VIP, and the sympathetic neurotransmitter norepinephrine. These neurotransmitters activate different signaling pathways to increase the tear secretion, although some parts of the pathways interact.

The signal pathway of Ach starts from its receptor, M3AchR, on the cellular membrane. (Figure 28) M3AchR stimulate the Gαq which activates the phospholipase Cβ (PLCβ). PLCβ breaks down into IP3 and PKCs that the IP3 is released into the cell plasma and PKCs stay in the membrane. IP3 binds to its receptor on the endoplasmic reticulum and releases Ca2+, so that the intracellular Ca2+ concentration is increased. The decrease of intracellular Ca2+ storage directs the incoming Ca2+ across the cell membrane. This Ca2+ influx maintains the high intracellular Ca2+ concentration as well as the Ca2+ storage. Ca2+ stimulates exocytosis by activating target proteins to release tears. Ach also activates inhibitory pathways that decrease tear secretion. Ach activates Pyk2 that causes activation of the MAPK pathway. The step that activates Ras is not clear. However, the activation of MAPK decreases the cholinergic agonist stimulated secretion, thus serving as a negative feedblack loop to control tear secretion. (Dartt 2009; Levin and Kaufman 2011)

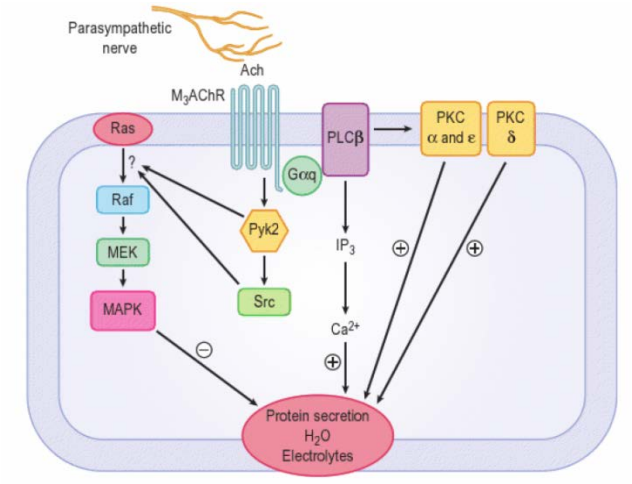

Figure 28: Schematic of parasympathetic signaling pathway with acetylcholine (Ach). (Levin and Kaufman 2011)

The signal pathway for VIP, which is released from parasympathetic nerves, is different from Ach pathway. (Figure 29) VIP binds to its receptor to activate the G protein, Gαs, that activates adenylyl cyclase (AC). AC then produces cAMP from ATP, and cAMP activates PKA which phosphorylates target proteins in the exocytotic process. The effects of VIP is increasing the intracellular Ca2+ as well, which promotes the protein and water secretion in the acinar cell. (Dartt 2009)

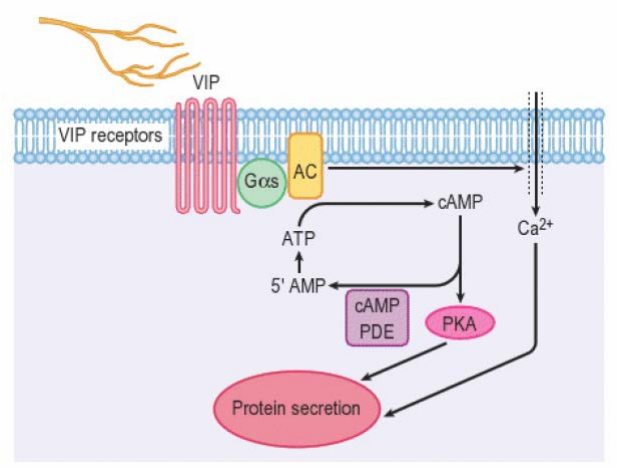

Figure 29: Schematic of VIP signal pathway for tear secretion.

8. Blinking

8.1 General information

The structures within the eyelid can be divided into two parts: the anterior part includes the skin, muscles and associated glands; the posterior part includes the tarsal plate, conjunctiva and associated glands (Figure 30). Different eyelid muscles are responsible for opening and closing the eyelids.

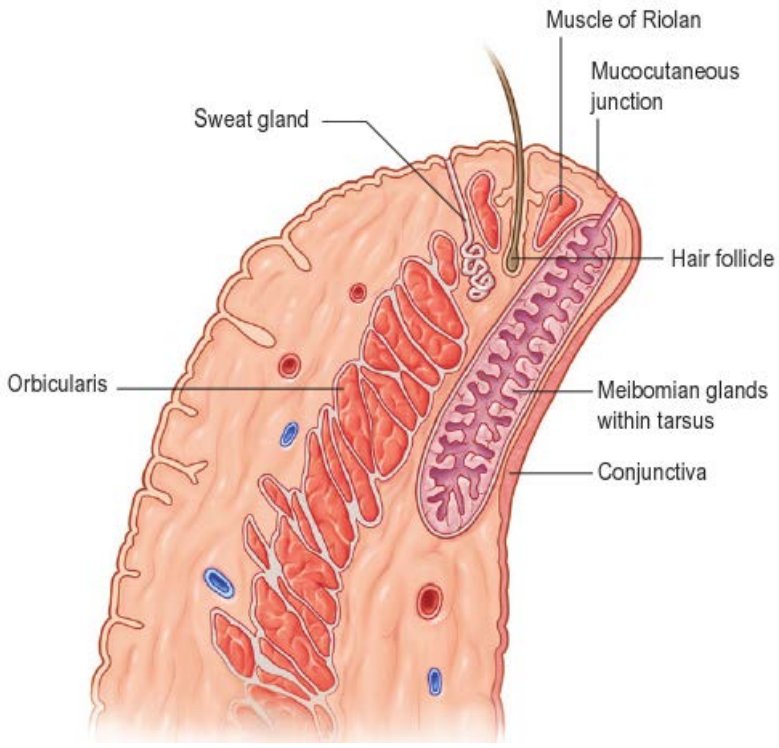

Figure 30: Diagram of the saggittal section of the eyelid. Left is the outside of eye, right is inside of eye. There are skin, muscles, tarsal plate and conjunctiva from the left to the right. (Levin and Kaufman 2011)

Opening the eye mainly depends on the levator palpebrae superioris (LP) muscle, as well as small contributions by the lower lid retractors and the smooth Muller muscles. Closing occurs by the contraction of the orbicularis oculi (OO) muscles, which surrounds the palpebral fissure circumferentially.

Blinking begins with the activation of the OO muscle (Figure 31). Under the normal conditions, the eyelid is lifted by the tonic activated LP muscles and the eye opens. Before it blinks, the activation of the LP muscles is shut down and the OO

muscles increase their activation; thus, shutting the eyelid. When the lid reaches its lowest point, the OO muscles deactivate, and the LP muscles take over, either increasing their activities above the normal level or returning to normal. Then the lid is pulled up, the eye is reopened.

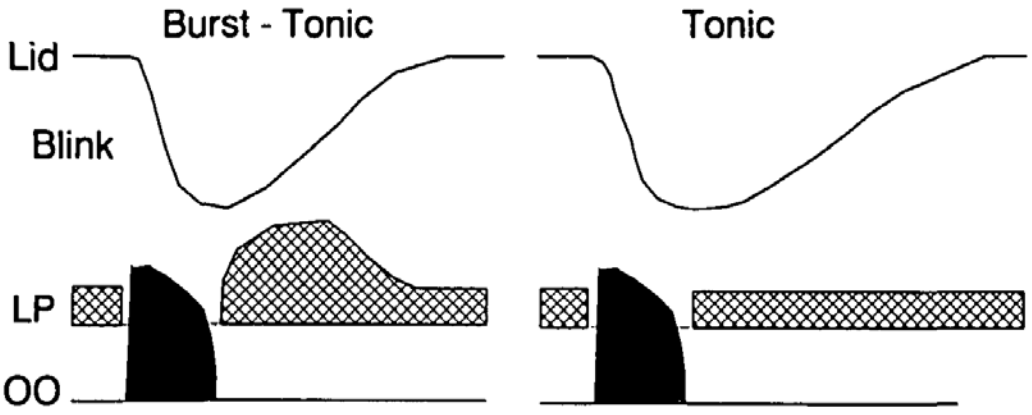

Figure 31: Hypothetical muscles activities and the lid movements within a blink. Lid: lid location, LP and OO: EMG signals from LP and OO muscles. If the LP muscle is over-activated during the up phase, this pattern is called Burst-Tonic pattern. If the LP muscle is back to its normal level during the up phase, this pattern is called tonic pattern (Evinger, Manning et al. 1991)

8.2 Eyelid Muscle Involved in blink

The main muscles involved in closing the eye are the OO muscles, which are innervated by the 7th cranial nerve. From an anatomical aspect, they are broadly divided into three parts: pretarsal, preseptal, and orbital parts (Figure 32). Viewed from the front,

the OO muscles cover the entire orbital opening, extending beyond the orbital margins onto the face. Bundles of muscle fibers run in elliptical shapes, and they become larger as the fiber bundles are located further from the margins. Individual muscle fibers do not run all the way between the medial and lateral palpebral ligaments. The fibers originating from one of the ligaments extend perhaps a third of the distance across the lid and insert into the connective tissue associated with the muscle. Fibers in the middle of the eyelids run toward the medial and lateral sides, but they never reach the ligaments, covering only the middle third of the eye length. These three segments of muscle bundles have different bundle lengths and are overlapping. Therefore, they might contribute to the variety of eyelid movements.

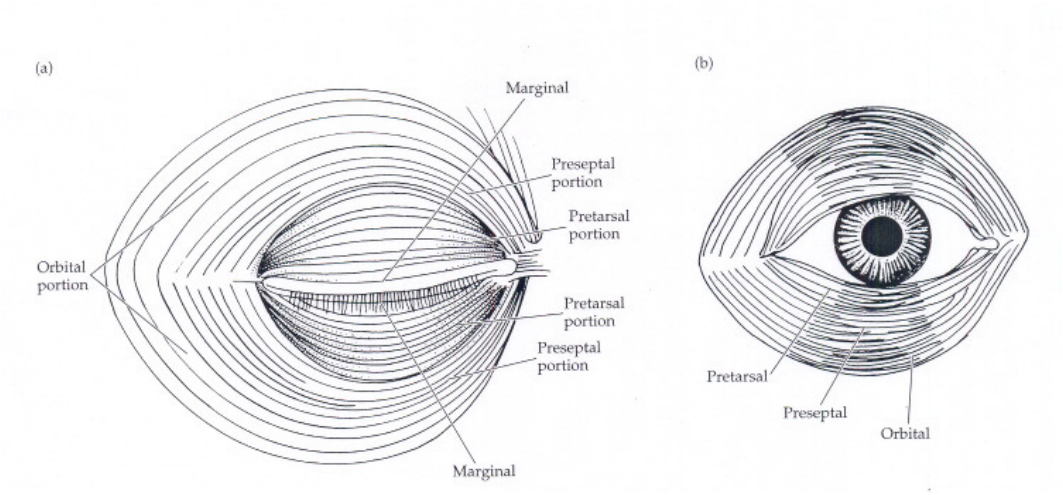

Figure 32: Diagrams of the front view of the OO muscles. The muscles are separated into three parts: pretarsal, preseptal, and orbital from the central to the peripheral areas. (Oyster 1999)

The levator muscle (LP), innervated by the superior division of the oculomotor nerve, takes the main responsibility in pulling up the eyelid, combined with the Muller muscles. The anatomy of the LP is showed in Figure 33. Since this action goes against gravity, the LP needs to stay activated to a certain level, even under non-blinking conditions. It is not clear how the OO and LP communicate with each other, switching their activation during the blink. If the LP muscle is over-activated during the up phase, there will be a faster up phase (burst-tonic pattern, see Figure 32), whereas if LP muscle is back to its normal level during the up phase, there will be a relative slow up phase (tonic pattern).

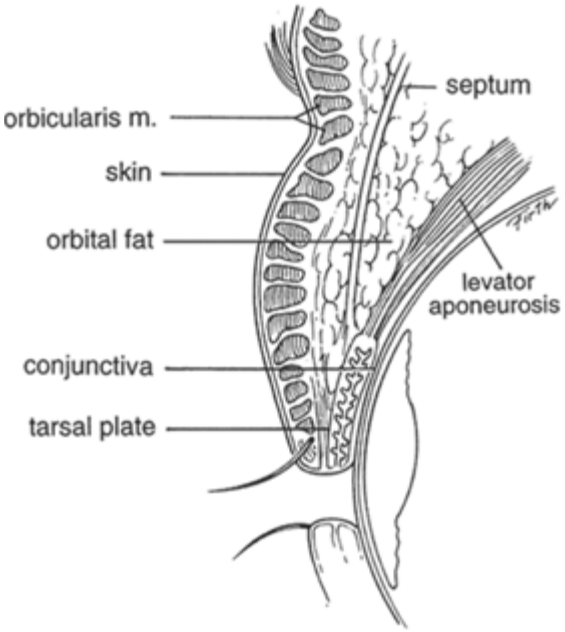

Figure 33: Diagram of the cross section of the upper eyelids. The levator muscles attaches on the top of the tarsal plant. Image from internet. (http://ovidsp.tx.ovid.com/sp-3.4.2a/ovidweb.cgi?&S=JKCNFPKBFFDDHJIINCBLEGDCJAAOAA00&Link+Set=S.sh.17|1|sl_10)

8.3 Neural pathway of Reflex Blink

Even though the neural pathway of spontaneous blinking remain unclear, (Kaminer, Powers et al. 2011) the neural circuit for reflex blinking has been outlined (Figure 34). There are three components within the pathway: the primary cornea afferents, the second order trigeminal complex neurons, and the facial motor nucleus which projecting on the orbicularis oculi muscles. (Henriquez and Evinger 2007).

The primary cornea afferents include the receptors on the cornea, the anterior ciliary nerves within cornea and conjunctiva, and the ophthalmic branch of trigeminal nerve. Neural signals pass through these structures and reach the trigeminal brainstem complex for its first projection. The sensory signals transfer from the trigeminal brainstem complex to the facial motor nucleus which controls the eyelid muscles for blink. Detailed information on how the cornea responds to surface stimuli was discussed earlier in the corneal innervation section.

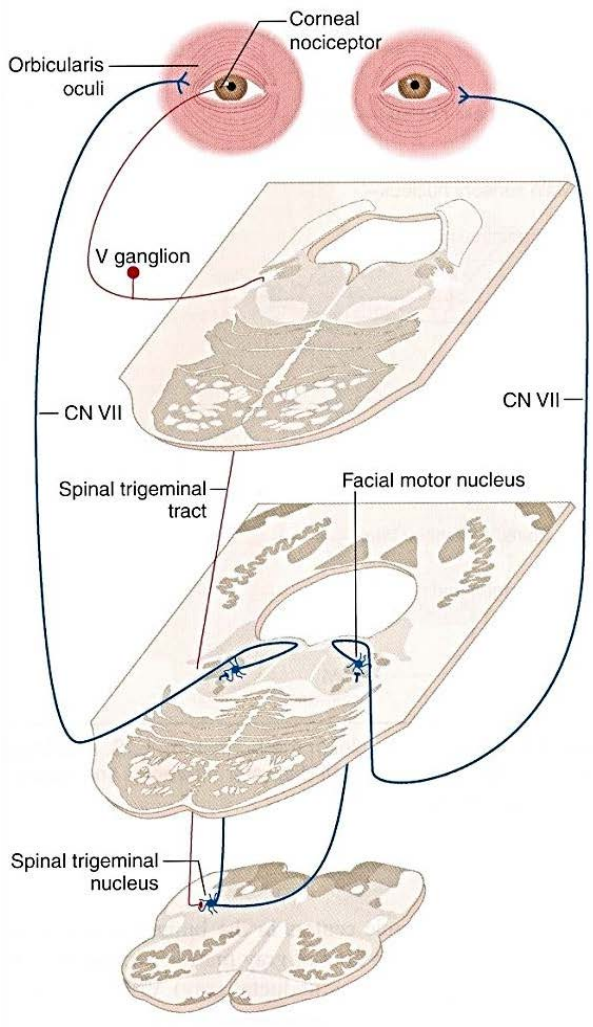

Figure 34: Diagram of the reflex blink circuit (Nolte, 2007)

8.4 Conditional blinking

Conditional blinking is a form of classical conditioning that has been studied to understand the neural mechanisms underlying learning and memory. The procedure is relatively simple. Two stimuli are provided: one is the conditioned stimulus (CS), which is auditory or visual; the other is the unconditional stimulus, which is a mild air puff or an

electric shock to the corneal nerve. Initially, the unconditional stimulus (UCS) can trigger a blink, whereas the CS cannot under normal conditions. When applying a UCS to a subject, it is always paired with a CS. After many repetitions, a learned movement is formed, whereby the blink is triggered by UCS. These conditional blinks can happen for a long time, but they can also disappear over time by only presenting the NCS without the CS.

This process has been studied in many mammalian species, such as the mouse, rabbit, guinea pig, cat and human. The neural structure and mechanism of conditional blinking is explained in Figure 35 (Medina, Repa et al. 2002). In this figure, it shows that the information from the CS pathway and UCS pathway converge into specific regions of the deep cerebellar nuclei (interpositus nuclei). This research on conditional blinking suggests that the eyelid can adjust its movement by learned responses, possibly changing normal blinking patterns.

The main purpose of the blink is to protect the cornea and maintain a healthy tear film. Signals from the corneal surface might modulate the blink rate and amplitude to avoid the irritation on the surface. However, dry eye irritation may produce over activated neural activities on the ocular surface, which may act as an error signal to adjust the blink amplitude and frequency, creating an adaptive process to the onset of benign essential blepharospasm. (Peshori, Schicatano et al. 2001; Evinger, Bao et al. 2002)

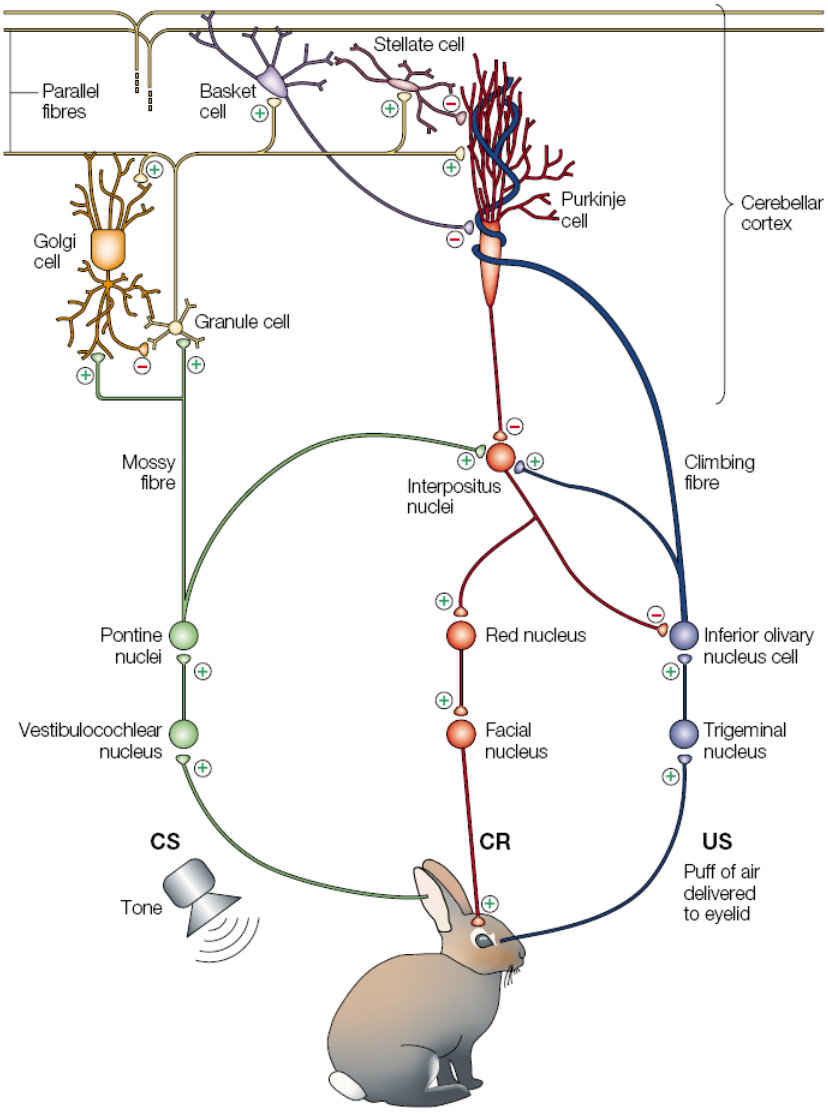

Figure 35: Diagram of the neual circuits during the eyelid conditioning (Medina, Repa et al. 2002). The blue line shows the unconditional stimulus pathway, and the green line shows the condional stimulus pathway. The red line shows the motor pathway.

8.5 Previous Studies on Blinking

8.5.1 Three Types of Blinks

Blinking activities are classified into three types: reflex, voluntary and spontaneous blinks, based on Evinger's observations on the characteristics of EMG signals from the orbicularis muscles. (Evinger, Manning et al. 1991) These three types of blinks were triggered in different ways. Reflex blinks can be experimentally induced by electrical stimulation of the trigeminal nerve, bright lights or loud sounds and by air puff stimuli to the ipsilateral or contralateral ocular surface. The voluntary blink occurs when subjects are asked to blink or they blink when hearing a tone. Spontaneous blinks are defined as all of the other blinks, often called natural blinks (Evinger, Manning et al. 1991).

Researchers have measured spontaneous blinks under many conditions, including asking subjects to relax (Agostino, Bologna et al. 2008), staring at eye level targets (Evinger, Manning et al. 1991), observing videos (Garcia, Pinto et al. 2011), playing computer games (Vajpayee, Thomas et al. 2000; Himebaugh, Begley et al. 2009; Jansen, Begley et al. 2010; Cardona, Garcia et al. 2011; Wu, Begley et al. 2014), while reading and in conversation (Doughty 2001).

8.5.2 Dopamine system on spontaneous blink

The spontaneous blink rate is widely used as a marker to assess central dopamine activities. Several studies found that low dopamine levels were correlated with a low blink rate, whereas an over-activated dopamine system induced a high blink rate (Karson

1983). For this reason, it is thought that the spontaneous blink rate decreases with Parkinson disease (Agostino, Bologna et al. 2008; Reddy, Patel et al. 2013), mental retardation and repetitive behavior disorders, (Goldberg, Maltz et al. 1987; Bodfish, Powell et al. 1995) progressive supranuclear palsy (Golbe, Davis et al. 1989; Bologna, Agostino et al. 2009), and recreational cocaine use (Colzato, van den Wildenberg et al. 2008). These conditions are all related to a low dopamine level. On the other hand, the blink rate increases with schizophrenia (Helms and Godwin 1985; Mackert, Woyth et al. 1990), psychosis (Karson, Goldberg et al. 1986) and fragile X syndrome, (Roberts, Symons et al. 2005) which are characterized by a high dopamine level.

8.5.3 Effect of mental task on spontaneous blink

Spontaneous blink activities vary significantly in different mental tasks. When engaged in visual display terminal tasks (playing computer games), the blink rate is significantly decreased and the interblink interval is significantly increased (Schlote, Kadner et al. 2004; Cardona, Garcia et al. 2011). Even when performing tasks requiring similar concentration, blink activity is influenced by the rate of visual information presented. In other words, the more visual information presented, the lower the blink rate (Cardona, Garcia et al. 2011). Partial blinking is very common while concentrating on a visual task such as a computer game (Jansen, Begley et al. 2010; Portello, Rosenfield et al. 2013; Chu, Rosenfield et al. 2014; Wu, Begley et al. 2014). Some studies suggest that partial blinking might due to poorly inhibitory blinks during a visually demanding task (Kennard and Smyth 1963; Portello, Rosenfield et al. 2013). The concentration task tries

to inhibit the blink occurrence, however this inhibitory effect is not strong enough to completely block the blink; therefore partial blink happens during the visual demanding task. The blink rate also decreases with nonvisual tasks, such as an auditory tracking task.(Gregory 1952) These studies suggest that cognitive concentration has a strong inhibitory effect over the internal system which sets the basal blinking activities (blink rate and amplitude etc.). Blink rate increases significantly during conversation and interviews (Doughty 2001). Since speaking is considered as a higher state of arousal compared with silent reading or computer work, it induces more blinks (Stern, Walrath et al. 1984). In summary, these results suggest that different mental activities can change the blink pattern dramatically.

8.5.4 Effects of ocular surface on spontaneus blink

Spontaneous blinking is an important protection for the ocular surface, as well as providing the mechanism for wetting as the lid is wiped over the ocular surface. A recent study suggests that the spinal trigeminal complex might be a major element in the spontaneous blink generator. It means that the basal level of corneal afferent input to the spinal trigeminal complex may establish the mean IBI for spontaneous blinking (Mutch 1944). Several clinical studies have shown that the blink rate is related to ocular surface conditions, such as wearing a contact lens. Wearing contact lens increases the blink rate for most subjects, even when playing a computer game, which implies that ocular surface stimulation can overcome the inhibitory effect of task concentration.(Jansen, Begley et al. 2010) For dry eye patients, who have poor tear film and many symptoms, the blink rate is

increased compared to controls (Vajpayee, Thomas et al. 2000). Similarly, blowing air in front of eye, increasing the evaporation and stressing the surface, increases the blink rate (Nakamori, Odawara et al. 1997). These results suggest that increasing ocular surface stimulation induces increased blinking. Conversely, using anesthetics decrease the blink rate significantly, but not the overall pattern of blinking pattern (Naase, Doughty et al. 2005; Borges, Garcia et al. 2010). In addition, using artificial tears (Nakamori, Odawara et al. 1997) or lubricated eye drops (Torkildsen 2009), which presumably smooth the surface and decrease ocular inputs can decrease the blink rate significantly.

However, some results conflict with each other. For example, some studies found a correlation between tear film breakup time and spontaneous blink rate (Prause and Norn 1987; Patel, Henderson et al. 1991; Al-Abdulmunem 1999), but others did not, (Schlote, Kadner et al. 2004) implying that tear film instability did not drive blinking. Others found that tear film breakup was not necessary for triggering a blink (Vajpayee, Thomas et al. 2000), therefore, the nature of how the ocular surface modulates the blink is not clear.

8.5.5 Temporal dynamics of blinking

The temporal pattern of the blinks is important since blinking maintains the corneal integrity(Varikooty and Simpson 2009) and the proper formation of the tear film.(Palakuru, Wang et al. 2007) Most previous studies mainly measured the blink rate,(Collins, Seeto et al. 1989; Bentivoglio, Bressman et al. 1997; Barbato, Ficca et al.

2000) however, the blink rate does not mean the time between each blink is constant. Indeed, under a controlled experimental condition, each individual appears to exhibit his own pattern of blinking, mixing interblink intervals (IBI) of shorter and longer duration.(Carney and Hill 1982; Freudenthaler, Neuf et al. 2003; Johnston, Rodriguez et al. 2013)

Under controlled experimental conditions, the blink pattern is highly variable, even within healthy subjects.(Doughty and Naase 2006) Doughty et al have suggested three types of IBI distributions: irregular, J-type and symmetrical, for healthy subjects while fixating a target in a quiet exam room.(Doughty 2002) (Figure 36) The irregular eyeblink pattern involved several fairly long IBIs interspersed with a couple of rapidly repeated blinks. Such rapidly repeated blinks result in very small IBI values, which mixed with long IBIs, lead to an overall high standard deviation of IBIs.(Figure 36A) The J-type showed some grouping of blinks, but the overall IBIs were more regular compared to the irregular type. An analysis of IBIs in J-type blinking showed a positively skewed histogram in which most of the IBIs were short with some longer IBIs.(Figure 36B) The regular type is symmetrical with blinks occurring at relatively constant intervals.(Figure 36C)

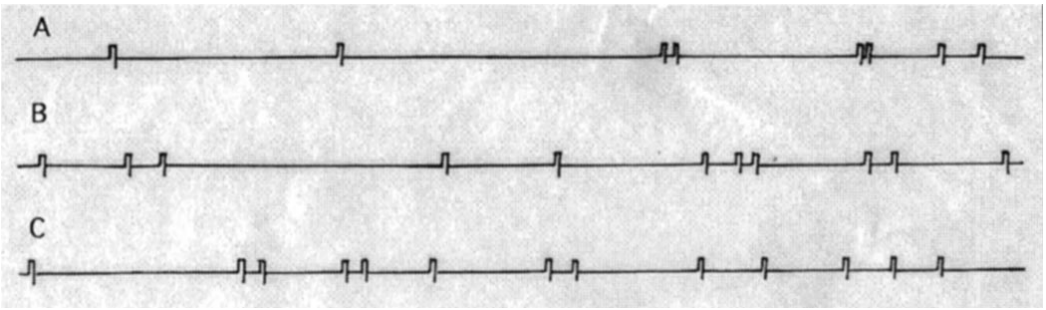

Figure 36: Three individual examples of blink event marker traces while fixing a target in a quiet exam room. A, irregular type; B, J-type; C, symmetrical type. (Doughty 2002)

9. Tear turnover rate

Traditional methods of measuring tear production are Schirmer (Lamberts, Foster et al. 1979) and Phenol red thread tests (Kurihashi, Yanagihara et al. 1977) that quantify the liquid absorption by using a small paper strip and cotton thread. However, both tests are poorly correlated with the tear production: the Schirmer test is a measure of reflex tear production, which has low specificity and sensitivity between normal and dry eye subjects (Lamberts, Foster et al. 1979); the Phenol red thread test has also been questioned about what it actually measures . Previous study has showed that the exact parameter measured with the cotton thread test may not be the real tear secretion volume but the liquid volume resides in the conjunctival sac. As a result, many studies have been performed to measure the rate of disappearance of fluorescein placed in the tear film. The result, which is called tear turnover rate, is the output combining both new tears production and tear drainage from the ocular surface. In most studies, the disappearance of fluorescein from the tear film has been used to record tear turnover rate by the

technique of fluorophotometry. (Xu and Tsubota 1995; Sorbara, Simpson et al. 2004; Mochizuki, Yamada et al. 2009; Chen and Ward 2010; McCann, Tomlinson et al. 2010)

A commercial fluorophotometer with analysis software has been developed to standardize the procedure of tear turnover rate.(van Best, Benitez del Castillo et al. 1995) The fluorescein concentration in the tear film is measured by this techniques over a period of 30min after instillation of 1µl of 2% fluorescein into the lower fornix; measurement is taken every one minutes. The change in fluorescence concentration is then calculated during the total measurement period and a biphasic decay in fluorescence is observed. The measurements in the first 5min show a rapid decay, which may be due to the instillation of fluorescein triggering the reflex tears. The later part of the curve (5min after instillation) represents the tear turnover under basal condition which shouldn't be affected by the initial lacrimation. Data in this time period is analyzed by the following equations to obtain the basal TTR: (Tomlinson, Doane et al. 2009)

TTR= [c(t0)- c(t0+1)]*100 / c(t0) (%/min) Equation 1

c(t0)= fluorescein concentration in the tear film at time t0 (min)

c(t0+1)= fluorescein concentration in the tear film one minute later after time t0

Assuming a monophasic decay of fluorescence from 5min post-instillation with a decay time constant b (/min); c(t)= c(0)* e^(b*t) Equation 2

Applying equation 2 into 1, therefore the tear turnover rate is:

TTR= [1- e^(b*t)]*100 (%/min) Equation 3

Above analysis gives a measurement of the TTR in %/min. In order to express the TTR in terms of flow with the unit of µl/min, it is necessary to either assume a value for the basal tear volume (typically 7µl) or to measure the volume from the initial dilution of the instilled fluorescein in the tears.(Mishima, Gasset et al. 1966) Initial dilution is calculated by back extrapolation to time zero of the initial fluorescence decay (Figure 37). In this technique, the fluorescence decay was treated as a linear relationship in the first 4 to 5min after instillation of the fluorescein. A second linear fit was performed after 5min.(Mishima, Gasset et al. 1966). With the estimated tear volume, the TTR can be converted from %/min into µl/min by tear volume times TTR in percentage.

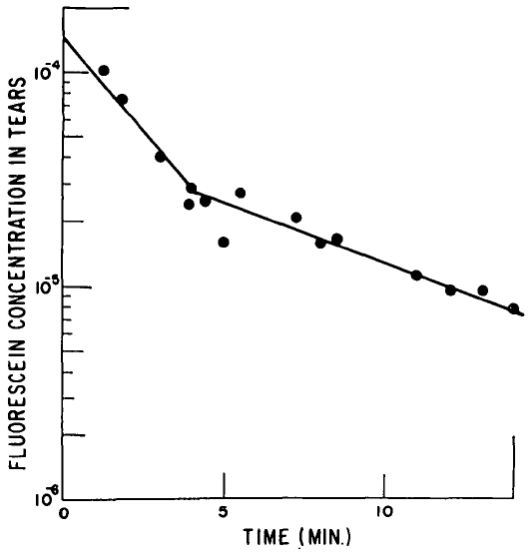

Figure 37: The fluorescein concentration change over time by Fluorophotometry (Mishima, Gasset et al. 1966). The initial concentration was calculated as the interaction with the y axis. The unit of fluorescein concentration (y axis) is grams per milliliter

Several studies have been performed the compared the TTR in normal and dry eye groups. (Sahlin and Chen 1996; Khanal, Tomlinson et al. 2009; McCann, Tomlinson et al. 2009; McCann, Tomlinson et al. 2010) The average normal tear turnover rate is 16.19±5.10%/min (1.03±0.39 µl/min), ranging from 10-20%/min. Dry eye subjects showed a decreasing TTR when used the same technique. The mean TTR was 7.71±1.02%/min (0.40±0.1 µl/min) in aqueous deficiency dry eye, and was 11.95±4.25%/min (0.71±0.25 µl/min) in evaporative dry eye. TTR is reduced 60% of the normal level in aqueous deficiency dry eye, and 30% in evaporative dry eye. (Tomlinson, Doane et al. 2009)

Tear turnover rate is a useful index for representing tear dynamics over the ocular surface. Stimulating the ocular surface will increase both blinking and tear secretion, increasing the tear turnover rate. However, the main issue with fluorophotometry is the low temporal resolution. As mentioned before, the fluorescein concentration can be measured every one or two minutes with a fluorophotometer, but reflex tearing will occur much faster; thus, it may not be able to track the rapid events that occur during the reflex tearing.

10. Lacrimal excretory system

The drainage of the old tear from ocular surface is done by the lacrimal excretory system, which involves the punctum, canaliculi, lacrimal sac, nasolacrimal duct and the inferior turbinate (Figure 38). The lacrimal excretory system extends from the lower and

upper puncta at the medial corner of the eyelids. The diameter is about 0.5 to 1mm. Tears drain though the lower and upper puncta into the canaliculi, flowing into the lacrimal sac. The canaliculi are thin ducts about 8- 10mm in length. They traverse the medial eyelids and enter the nasolacrimal sac at a 58 degree angle. This angle prevents the tears from flowing backward. In addition, the canaliculi are surrounded by the orbicularis muscle which when contracting squeezes the canaliculi. The total length of the lacrimal sac and duct is about 24mm, and 4-8mm anteroposteriorly. It finally guides the tears into the inferior turbinate in the nose. A mucosal flap, Hasner's valve, may be present at the opening of the duct into the nose. It prevents the fluid from flowing backward. (Kaufman, Alm et al. 2003)

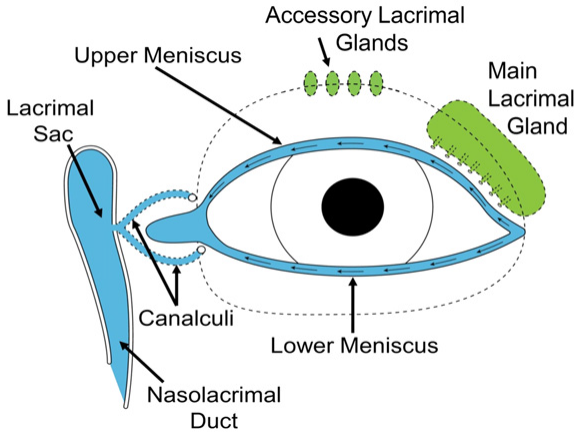

Figure 38: Diagram of the lacrimal excretory system. Tear is secreted from lacrimal, and finally drained into the nose. (Gaffney, Tiffany et al. 2010)

The most important mechanism of lacrimal drainage has been studied and described in several studies.(Maurice 1973; Wilson and Merrill 1976; Doane 1981) The

tear drainage is mainly due to the pressure gradient between menisci and canaliculi created in the blink cycle. The detailed information is described in Figure 39.

At the start of the blink, the canaliculi are already filled with tear fluid. At the beginning of blinking down phase, the upper lid goes down while the lower lid slightly elevates. The upper and lower puncta meet forcefully when the upper lid is about halfway of the eye aperture. The puncta remain blocked while the upper lid is still going down. The remaining portion of the lid closure compresses the canaliculi and lacrimal sac, squeezing the fluid into the nose through nasolacrimal duct. Thus, when the upper lid reaches its lowest point, the volume of fluid within the canaliculi is at its minimum and pressure is at its maximum. When the lid starts going up, the compressive force releases and the elastic walls of the canaliculi and sac tend to be back to their normal volumes. The pressure within canaliculi and sac drop down quickly, creating a negative pressure. The valves within the canaliculi and sac prevent the fluid drawn back into the system. Since the puncta are still blocked during the beginning of the blinking up phase, the pressure within canaliculi and sac gets more negative and reach to its maximum negative value right before the puncta open. Then, the puncta are suddenly open when the lid moves about halfway of the eye aperture. The pressure gradient sucts the fluid rapidly from the menisci into the canaliculi. Thus, the system is once again filled with fluid and ready for the next blink cycle.

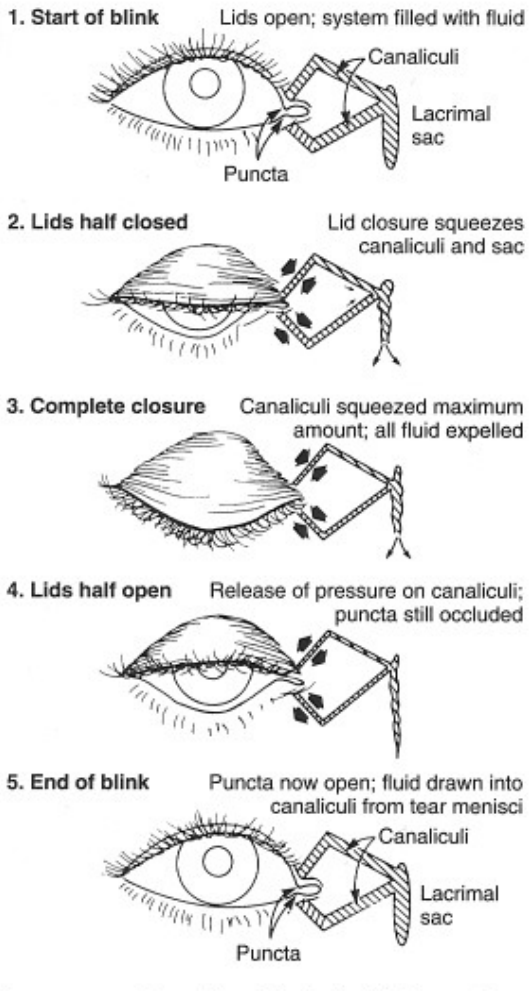

Figure 39: Diagram of the hypothesis model of tear drainage during a blink. (Kaufman, Alm et al. 2003)

11. Tear meniscus

The tear meniscus is formed between the lid surface and cornea or bulbar conjunctiva and is located along the eyelid margin. It has been estimated that the tear liquid is stored in the three compartments on the ocular surface: conjunctiva sac, menisci

and cornea. About 3/7 of the tears are stored in the sac, 3/7 in the upper and lower menisci and 1/7 is spread on the ocular surface. (Mishima, Gasset et al. 1966) Therefore, examination the tear meniscus is useful for clinician to estimate the tear volume. (Mainstone, Bruce et al. 1996; Tung, Perin et al. 2014)

Generally, clinicians and researchers only study the lower meniscus, since 1) there is less movement with blinking, 2) it is less blocked by eyelashes, and 3) the gravity effects on the meniscus formation is very small so that the upper and lower menisci are really similar. (Miller, Polse et al. 2002) The most common metric of tear secretion studis is tear meniscus height (TMH). There are two main techniques for the measurement of TMH, direct measurement with a reticle on the slit lamp or from a video recording, and the optical coherence tomography (OCT). (Johnson and Murphy 2005; Savini, Barboni et al. 2006; Garcia-Resua, Santodomingo-Rubido et al. 2009) The video recording can be performed with fluorescein to aid in visualization of the meniscus.(Kawai, Yamada et al. 2007)

The TMH can be measured simply using a slit lamp biomicroscope with a graticule on the eyepiece. However, this method is contaminated by eyes movement and measures relatively large increments.(Johnson and Murphy 2005) In addition, it requires an observer to make an immediate, one-time judgment. Therefore, it is of limited usefulness in research. The video recording method of TMH allows measurement of the TMH using a ruler by an observer unaware of the subject information, so that is less subjective, but still hampered by observer error. In order to analyze the image more objectively, it is possible to instill fluorescein dye to improve the contrast between the meniscus and its background for quantitative measurement.(Newsome, Gross et al. 1982)

Another widely used technique to measure TMH or volume is OCT, since it is non-invasive (no fluorescein instilled) and more tear meniscus parameters can be measured, including tear meniscus height, depth and area.(Eisenstein, Sorgente et al. 1973; Palakuru, Wang et al. 2007) However, the OCT image tends to be very noisy, and can be difficult to analyze the tear meniscus automatically. In addition, it is impossible to differentiate the initial and newly secreted tears on OCT, other than indirectly by increased TMH; therefore, it is less useful for the studying the tear turnover rate than using fluorescein dye.

12. Correlation between fluorescein concentration and intensity

Sodium fluorescein dye has been widely used clinically to visualize the tear film for more than 50 years. (Norn 1969) Several clinical tests, including tear breakup time and tear turnover rate, require fluorescein instillation. (Lemp and Hamill 1973; Occhipinti, Mosier et al. 1988) Thus fluorescein is important in accessing tear film and tear dynamics on the ocular surface.

Despite its clinical importance and usage for viewing the tear film, many properties of fluorescein molecules, such as concentration quenching, are rarely discussed in the ophthalmic literature. Concentration quenching is often used in cell biology to detect membrane permeability and cellular volume changes. (Hamann, Kiilgaard et al. 2002; Solenov, Watanabe et al. 2004) The reduction of the cell volume increases the dye concentration in the plasma, changing the dye intensity. Concentration quenching is

mainly due to the energy absorption by adjacent molecules, leading to an overall low intensity. Previous study has induced this concept to tear film that the fluorescein intensity reaches its maximum at 0.1% concentration when considering the tear film thickneiss is 5μm. (Figure 40) Fluorescein intensity drops down above and below this concentration.(Webber and Jones 1986) This work suggested that both very low and high concentrations of fluorescein will be too dark to visualize in the tear film, and should be avoided in clinical practice.

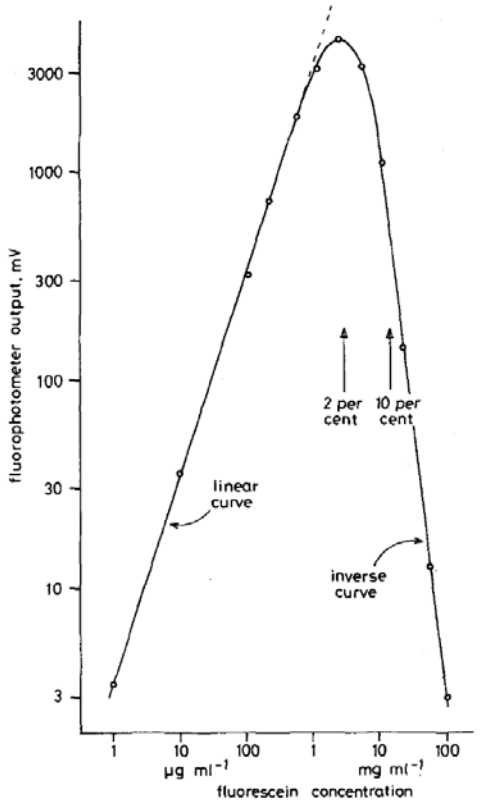

Figure 40: The correlation between fluorescein concentration and intensity. The fluorescein intensity was recorded from Fluorophotometry. The film thickness was approximately 5μm. (Webber and Jones 1986)

Recently, a mathematical model predicting the fluorescein concentration from fluorescence intensity in the tear film has been developed.(King-Smith, Nichols et al. 2008) Assuming both incident light and film thickness are constant, fluorescein intensity is dependent on two factors: 1) the illuminant absorptance, which is the fraction of the incident light absorbed by the fluorescein molecules, leading to molecular excitation. The illuminant absorptance is increased with fluorescein concentration, and saturated at certain concentration. 2) the fluorescein efficiency, which is the fraction of the excited fluorescein molecules which can emit the fluorescein light out of the solution. Fluorescein efficiency is decreased markedly with high fluorescein concentration, since the light or energy emitted from the bottom region is absorbed by the molecules in the adjacent or upper region. Thus the overall intensity is low (concentration quenching). (Figure 41)

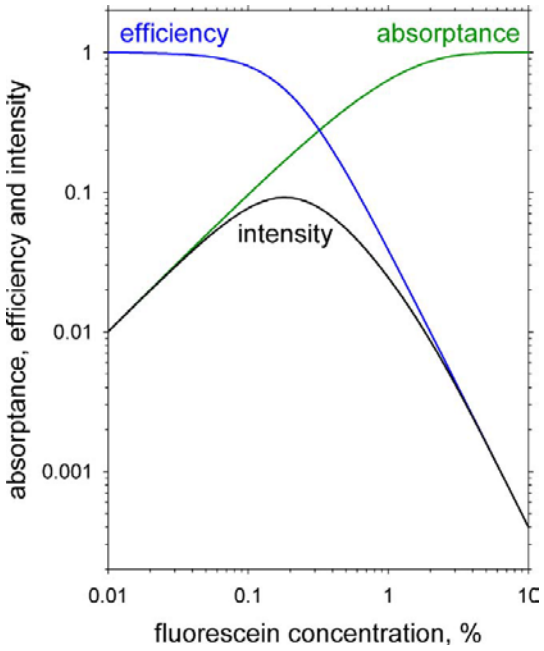

Figure 41: The mathematical model between fluorescein concentration and intensity. The film thickness was set as 5μm. (King-Smith, Nichols et al. 2008)

The equation between concentration and intensity is as follows:

$I(c)= k^* A(c)^* E(c)$ Equation 1

where I: fluorescein intensity, c: fluorescein concentration, k: constant (equals to 1) , A: absorptance, E: efficiency.

The absorptance is given by Beer's Law:

$A(c)= 1- T(c)= 1- e^{-\varepsilon cd}$ Equation 2

Where T(c) is transmittance, ε is the molar extinction coefficient, c is molar concentration of the fluorescein and d is the film thickness.

Efficiency of fluorescein is given by resonance energy transfer using the formula:

$E(c)= 1/(1+ (c/c0)^2)$ Equation 3

Where c0 is the critical concentration (equals to 0.2%) (King-Smith, Nichols et al. 2008)

Since tear film hyperosmolarity is the core mechanism of dry eye development, this model can be used to estimate the fluorescein concentration, which is related to fluid osmolarity, from the fluorescein intensity. (Braun, Gewecke et al. 2014) It is important to note that the film thickness is critical for the final intensity. Generally, thicker films show higher intensities than thinner films. Since both film thickness and concentration will be changed during the evaporation, this model can be used to understand the fluorescein

intensity dynamics within tear film breakup or thinning. It can also theoretically be used to calculate fluorescein concentrations in the menisci.

Summary

In summary, dry eye is a common condition that affects millions(Schaumberg, Sullivan et al. 2003; Schaumberg, Dana et al. 2009) and any disturbance in the lacrimal functional unit might contribute to the development of dry eye.(2007) In this introduction, I have reviewed each component in the lacrimal functional unit, including tear film, ocular surface, blinking, tear secretion and the underlying neural system. Blinking and tearing are the two automatic responses triggered by the signals on the ocular surface,(Nakamori, Odawara et al. 1997; Situ and Simpson 2010) and their quality and quantity are critical for maintaining a healthy surface.(Harrison, Begley et al. 2008; Cardona, Garcia et al. 2011; Hirota, Uozato et al. 2013) Despite the importance of blinking and tearing in protecting and maintaining a normal ocular surface, few studies explore their dynamic activities under conditions of varying surface stimulation. In this thesis, I applied measurable ocular surface stimuli and quantified the blinking and tearing responses to better understand their activities.

In the first project,(Wu, Begley et al. 2013) I monitored the detailed blink parameters, including the frequency, amplitude, velocity and duration, in response to a relatively mild surface stimulation while subjects performed high and low concentration tasks. I found that doing a visual concentration task and having a mild surface

stimulation had opposite effects on blink frequency and duration. Engaging a high concentration task decreased the blink frequency and duration,(Schlote, Kadner et al. 2004) presumably to minimize the lid interruption. Having a mild surface stimulation will increase the blink frequency and duration,(Tsubota, Hata et al. 1996; Acosta, Gallar et al. 1999) presumably to protect the ocular surface. Subjects with dry eye symptoms tended to have more individual variation compared to normal, presumably due to their unstable tear film as well as the abnormal nerve function on the ocular surface. There was a poor correlation between tear breakup area and blink parameters, thus the nature of ocular surface controls over blink was still unclear.

In the second project,(Wu, Begley et al. 2014) I further explored the ocular surface controls over blinking when gradually increased the surface stimulation. The task attention was controlled as much as possible by having subjects play a computer game. Both blink frequency and regularity were increased with surface stimulation, confirming the effects of ocular surface inputs on blinking in the first project. Moreover, I found a linear relationship between the stimulus intensity and the blink response after log transformation, suggesting neural system that could both detect external stimuli and appropriately modulate the blink activity, presumably to protect ocular surface from dangers. In addition, there was a high correlation between blinking and some ocular surface sensations, which highlighted their common input from the ocular surface.(Stapleton, Marfurt et al. 2013)

In the third project, I investigated tear secretion as well as blinking to better understand these auto-protective responses, while varying the surface stimulation. New

metrics based on the tear meniscus fluorescein concentration were developed to better study the tear dynamics in the lower meniscus. As expected, both blink and tear secretion were increased to protect the surface.(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Situ and Simpson 2010) After log transformation, both blink and tear secretion were increased linearly with surface stimulation, suggesting a robust nerve system in healthy subjects.(Wu, Begley et al. 2014) Interestingly, neither blinking nor tearing responses(Holly, LauKaitis et al. 1984) were constant during the air stimulation. After transient increments, both responses were decreased and maintained at certain levels which were proportional to the surface stimulation.

As in the previous project, the blink was increased linearly, whereas the tear secretion was increased exponentially.(Holly, LauKaitis et al. 1984) It is possible that blinking and tearing may be controlled by different types of neurons on the ocular surface, leading to their different responses. Both blinking and tear secretion showed adaptation to the stimulus, which may be related with underlying neural discharge rates showing adaptation over time.(Gallar, Pozo et al. 1993; Chen, Gallar et al. 1995; Parra, Madrid et al. 2010) In addition, newly secreted tears appeared a few seconds later than the blink response, which might be related to the tear transportation pathway from the lacrimal gland to the lower meniscus.(Gaffney, Tiffany et al. 2010) These findings may be helpful for understanding the physiology of the lacrimal functional unit and can be applied in studying dry eye etiology in future.

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CHAPTER II

THE EFFECTS OF MILD OCULAR SURFACE STIMULATION AND CONCENTRATION ON SPONTANEOUS BLINK PARAMETERS

Abstract

Purpose: To explore the interaction between ocular surface sensory and cognitive controls on multiple blink parameters.

Methods: Ten subjects participated in this study. There were 2 visits, one with an ocular surface air stimulus (AS) and one without (NS). The AS was set at a level barely perceptible by subjects (approximately 0.6m/sec at the eye). At each visit, subjects performed a high (HC) and low concentration (LC) task. Blinking was tracked and tear-film breakup (TBU) was monitored simultaneously to measure blink parameters, including the interblink interval (IBI), blink amplitude, duration, maximum velocity and TBU before and after each blink.

Results: During the HC task, IBI was significant longer and blink duration was shorter (repeated measures ANOVA, $p< 0.05$) than the LC tasks. When having surface stimulation, IBI was shorter and blink duration was longer during the AS-LC condition compared to NS-HC condition. (Hotelling T^2 test, $p<0.005$). There was high individual

variation in correlations between blink amplitude and maximum velocity. The area of TBU was not significantly correlated with any blink parameter.

Conclusions: The lack of correlation between TBU and blinking suggests that many blinks are stimulated by internal controls, rather than direct stimulation of the ocular surface by TBU. This pilot study suggests that even very mild ocular surface stimulation produces opposite effects on the timing and duration of the blink, when compared to concentrating on a visual task. The HC task tends to decrease blink frequency and duration, presumably to minimize interruption by the eyelids, whereas mild ocular surface AS increased blink frequency and duration, most likely to increase protection of the ocular surface.

This project has been published in Current Eye Research.(Wu, Begley et al. 2013)

Introduction

Dry eye is a common condition that affects millions in the US(Schaumberg, Sullivan et al. 2003; Schaumberg, Dana et al. 2009) and worldwide.(McCarty, Bansal et al. 1998; Uchino, Nishiwaki et al. 2011) It is considered to be a multifactorial disease of the tear film and ocular surface and is associated with symptoms of ocular discomfort and visual disturbance.(2007) Low blink rate has been identified as a potential risk factor for the development of dry eye because it can result in increased evaporative loss from the tear film.(Tsubota and Nakamori 1995; Nakamori, Odawara et al. 1997; 2007; Ousler, Hagberg et al. 2008; Himebaugh, Begley et al. 2009) This in turn may lead to increased tear film hyperosmolarity and instability,(Liu, Begley et al. 2009) which are considered core mechanisms of dry eye.(2007) In addition, the blink acts to spread the tear film over the ocular surface,(Oyster 1999) so that understanding its interaction with the tear film and the stimuli involved in the blink response may be important in the etiology of the dry eye condition.

Controls over spontaneous blinking, which include all but voluntary or reflex blinks,(Evinger, Manning et al. 1991) remain controversial.(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Naase, Doughty et al. 2005) Blink rate is known to be highly variable and depends on many factors, including cognitive state,(Bentivoglio, Bressman et al. 1997; Doughty 2001; Hirokawa, Yagi et al. 2004) ocular surface input(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Doughty, Naase et al. 2009) and central dopamine level.(Karson 1983; Barbato, De Padova et al. 2007; Agostino, Bologna et al. 2008) Visual tasks requiring concentration, such as playing

computer game or reading are known to slow the blink rate,(Acosta, Gallar et al. 1999; Doughty 2001; Himebaugh, Begley et al. 2009; Cardona, Garcia et al. 2011) sometimes markedly,(Schlote, Kadner et al. 2004) whereas stimulation or irritation of the ocular surface can increase the blink rate.(Nakamori, Odawara et al. 1997; Himebaugh, Begley et al. 2009) The ocular surface sensory controls over blinking is of special interest due to its putative effect in dry eye,(Palakuru, Wang et al. 2007) where sensory input from the unstable tear film(Liu, Begley et al. 2009) and (or) corneal sensory nerve damage or functional changes may be expected to affect blinking.(Peshori, Schicatano et al. 2001; Belmonte, Acosta et al. 2004; Toshida, Nguyen et al. 2007; Kaminer, Powers et al. 2011)

Many of the studies investigating the effect of the ocular surface on human blink rates have involved relatively dramatic or sizeable changes in ocular surface input, such as abrogating ocular surface sensation with anesthetic, which decreases the blink rate.(Nakamori, Odawara et al. 1997; Naase, Doughty et al. 2005; Borges, Garcia et al. 2010) Likewise, damage to corneal nerves following refractive surgery is associated with a lower blink rate,(Toda, Asano-Kato et al. 2001) whereas placing a contact lens on the eye, which could be considered an irritant to the ocular surface, has been reported to increase the blink rate.(Jansen, Begley et al. 2010) The dry eye condition is associated with an increased blink rate,(Tsubota, Hata et al. 1996; Himebaugh, Begley et al. 2009) perhaps due to stimulation by the unstable tear film,(Himebaugh, Begley et al. 2009) although few studies have investigated the connection between the tear film and blinking or devised experimental conditions with a relatively mild stimulus to the ocular surface.

Recently, it has been suggested that the spinal trigeminal complex plays a key role in neural circuit responsible for generating spontaneous blinking, by acting to integrate signals from the ocular surface and basal ganglia.(Kaminer, Powers et al. 2011) While it is clear that substantial blockage, stimulation or alteration of corneal afferents by instilling an anesthetic,(Nakamori, Odawara et al. 1997; Naase, Doughty et al. 2005; Borges, Garcia et al. 2010) wearing a contact lens(Jansen, Begley et al. 2010) or neural damage(Toda, Asano-Kato et al. 2001) are sufficient to effect changes in blinking, little is known about the effect of lesser ocular surface stimuli on blinking in humans. For this reason, we designed a pilot study to explore the effects of a relatively mild air stimulus to the ocular surface while varying the level of attention to a visual task with simultaneous monitoring of tear film instability to examine any associated changes in blinking. In addition, we investigated multiple blink parameters, rather than focusing on blink rate only, because factors such as blink amplitude and duration may also be affected by attention or concentration on a visual task(Jansen, Begley et al. 2010; Cardona, Garcia et al. 2011) and ocular surface stimulation.(VanderWerf, Brassinga et al. 2003)

Methods

1. Subject

The study was conducted at the Borish Center for Ophthalmic Research at the Indiana University School of Optometry, Bloomington, Indiana and adhered to the tenets of the Declaration of Helsinki. It was approved by the Institutional Review Board at

Indiana University and informed consent was obtained from each subject prior to beginning the study.

Ten subjects, some with and some without dry eye symptoms, as measured by the Dry Eye Questionnaire (DEQ),(Begley, Chalmers et al. 2003) were recruited for this study. We recruited subjects with a range of dry eye symptoms because this pilot study was exploratory in nature, with an eventual, future goal of studying the dry eye condition. However, subjects who showed significant corneal staining (>Grade 2, Oxford Scale(Bron, Evans et al. 2003)) were excluded because corneal staining could potentially be a confounding factor affecting the ocular surface sensory response to tear film instability.(Belmonte, Acosta et al. 2004; De Paiva and Pflugfelder 2004; Situ, Simpson et al. 2008) Subjects with ophthalmic conditions other than dry eye or systemic disease were also excluded.

Before beginning the study, subjects were informed that the experiment was designed to observe the tear film while they were doing tasks. Monitoring of blinking was not disclosed until subjects completed the study to prevent self-conscious or unnatural blinking.

2. Experimental Procedures

This study consisted of two visits: with (AS) and without (NS) an air stimulus. In order to also vary the level of attention to a task, each subject performed a high (HC) and low concentration (LC) task at each visit. The order of the visit and task (AS-HC, AS-

LC, NS-HC and NS-LC) was randomly determined. The LC task consisted of listening to classical music while looking straight ahead. The HC task involved playing a computer video game (Tetris®), which the subject viewed through a beam splitter. Each task lasted 2.5 minutes and there was a 15-minute break between the tasks.

The AS was generated using a small electronic fan. The air stimulus level was set based on the results of a small pilot study with 25 subjects in which the distance of the fan to the eye was altered until it was perceived as a barely detectable light breeze that was not considered bothersome. An average final distance of 50cm from the eye was chosen, which yielded a measured air velocity of 1.34 mph (0.6m/s) at the eye. According to the Beaufort scale for wind speed (http://www.spc.noaa.gov/faq/tornado/beaufort.html), which includes categories 0 (calm) to 12 (hurricane), the velocity we used is at the low end of the "light air" category 1 (0.3-1.5m/s) and is insufficient to move wind vanes or tree leaves. It is considered to be very mild, even less than a "light breeze" (category 2).

After filling out the DEQ,(Begley, Chalmers et al. 2003) subjects were seated behind a Zeiss biomicroscope system (8**x** magnification) with two custom-fitted cameras, which were used to simultaneously record upper lid movement (Point Grey Research, 250Hz) and tear film stability (Mistubishi HS-U69, 30Hz). In order to track eyelid positions during blinking, a 2mm diameter reflective white dot (3M®) was positioned on the margin of the right upper lid. Two microliters of 2% fluorescein dye was instilled into the inferior bulbar conjunctiva using a micropipette for an initial grading of corneal staining (Oxford Scale(Bron, Evans et al. 2003)) and tear film stability assessment during

the experiment. At the air stimulus visit, the recording was started one minute after the onset of air stimulus to allow subjects become familiar with the stimulus. Only the right eye was tested and the left was held shut by the subject to ensure that stimulus from the ocular surface arose from the tested eye.

At the end of the study, the Schirmer's I tear test (without anesthetic) and fluorescein tear break up time (TBUT) were performed to assess the level of dry eye for study subjects.

3. Blink Analysis

From each trial, a one-minute recording was extracted for analysis that began at 30 seconds into the 2.5 minute task. During each blink, the vertical movement of the reflective tape on the upper eyelid was tracked through the blink process and parameters including blink amplitude, maximum velocities and durations of the down and up phases, and total duration were calculated using a custom MATLAB® (The Mathworks™, Natwick, MA) program. For each subject, the amplitude of a complete or 100% blink was calibrated prior to each task by asking the subject to blink fully and measuring the maximum vertical closure of the eyelid during that blink. The amplitude of all subsequent blinks for that subject was expressed as a percent ratio of the original, calibrated, full blink. The maximum velocity was calculated as the fastest eyelid movement during the down and up phases and the absolute maximum velocity was calculated in millimeters per second. When comparisons were made with blink amplitude, which was a relative measure calibrated by each individual's full blink, the

maximum velocity was also standardized by a calibrated full blink and expressed as a percent ratio of the full blink per second. The blink duration was defined as beginning when the lid velocity first reached 10% of its maximum velocity during the down phase and ended when it reached 10% of its maximum velocity during the up phase.(Bologna, Agostino et al. 2009) Basing on previous studies,(Evinger, Manning et al. 1991; Tsubota, Hata et al. 1996) the average blink duration was around 200-300msec, thus the frame rate (250 frames/sec) used here was sufficient to catch the eyelid movement. The interblink interval (IBI) was calculated as the time from the fullest extent of one blink to the fullest extent of the next blink.

4. Tear Breakup Analysis

Images of the tear film (single frame) immediately before and after each blink were obtained from the digital recordings to calculate the area of tear break-up (TBU) as a percent of the exposed corneal area before and after each blink using methods described previously.(Begley, Himebaugh et al. 2006; Liu, Begley et al. 2006; Himebaugh, Begley et al. 2009) Briefly, color fluorescent images were converted to grayscale, and the region of total exposed cornea (region of interest) outlined. A custom MATLAB® program was used to calculate the percentage of TBU using a threshold pixel intensity set by the investigator for each trial. The investigator was unaware of the subject or trial information.

5. Statistical Analysis

Repeated-measures ANOVA with Bonferroni corrected post hoc testing were used to test attention and air stimulus main effects and their interaction for all blink parameters. Hotelling T^2 tests were used to make multivariate comparisons of mean and standard deviation metrics of the IBI, blink amplitude and down phase duration during study conditions. A paired t test was used to compare the area of TBU before and after blinks. The Pearson's correlation coefficient was used to determine correlations between blink parameters and with the area of TBU.

Results

1. Subject

The average age (±standard deviation) of study subjects was 34±14 years (range: 22 -57 years). Six were female and 4 were male. The median DEQ-5 score(Chalmers, Begley et al. 2010) was 9 (range: 0-16), and half of the subjects reported a previous dry eye diagnosis on the DEQ.(Begley, Chalmers et al. 2003) Initial corneal and conjunctival staining was negligible in all subjects, with a median of 0 and a range of 0-1 on the Oxford Scale.(Bron, Evans et al. 2003) The average (±standard deviation) Schirmer's I tear test and TBUT were 17.5±5.9mm/5min (range: 8-24mm) and 11±10 sec (range: 2-58 sec), respectively. Given the combination of symptoms and clinical signs, previously

diagnosed dry eye subjects would be categorized as mild to moderate (≤ Grade 2), according to the DEWS modified Delphi guidelines.(2007)

2. Individual Example

Figure 1 displays all blinks during the 60 sec trial for Subject 1, who did not report a previous dry eye diagnosis (DEQ-5 score=0, Schirmers= 20mm/5min, TBUT=58sec). Under baseline conditions (Figure 1A), blinks were relatively similar and regularly spaced, although none were full amplitude. In comparison, increased concentration (Figure 1B) resulted in fewer blinks of lesser amplitude, with an increased IBI variability, while the air stimulus (Figure 1C) increased the amplitude and regularity of blinking. The combination stimulus (Figure 1D) produced a greater range of blink amplitude and duration and an increased IBI variability.

Subject 2, who had been previously diagnosed with dry eye (DEQ-5 score=16, Schirmers= 8mm/5min, TBUT= 3sec), displayed fuller blinks than Subject 1 under most conditions (Figure 2). As with Subject 1, blinking slowed with concentration (Figure 2B), increased with the air stimulus (Figure 2C), and the IBI became more irregular, with more incomplete blinks of shorter duration with the combination stimulus (Figure 2D). Some blinks, often categorized as cluster blinks(Doughty 2002) or blink oscillations(Peshori, Schicatano et al. 2001; Evinger, Bao et al. 2002) occurred very close together (Figure 2: closed arrows). Some blink traces were greater than 100% amplitude (Figure 2: open arrows), as defined by our measurement calibration system. These blinks

appeared to be due to the subject squeezing the eye tightly shut, perhaps in response to the irritation of the air stimulus, which exceeded the original calibration blink.

3. Blink Parameters

3.1. Interblink Interval (IBI)

The IBI under baseline conditions (NS-LC) can be converted to an average blink rate of 12 blinks/min, which is well within the range of previous studies.(Doughty 2001; Himebaugh, Begley et al. 2009) Regardless of the AS, the average IBI under HC was significantly increased compared to LC ($p= 0.017$). In comparison, AS appeared to decrease the IBI regardless of concentration levels, although the difference did not reach statistical significance ($p=0.067$). There was no interaction between concentration and air stimulus main effects ($p>0.05$), suggesting that the effect of doing a high concentration task on IBI did not depend on the level of ocular surface stimulation. As Figure 3A shows, IBI varied within and among subjects under most of the experimental conditions except for the AS-LC condition under which variability was relatively low.

As Figure 3 shows, both the mean and within subject variability of the IBI appeared to be affected by the conditions tested in this study. Figure 4A plots the relationship between the mean and standard deviation of the IBI, comparing the effect of concentration (NS-HC) to the air stimulus condition (AS-LC). There was little overlap between the points, with the AS-LC points clustered at the bottom left of the graph due to their short IBIs and low variability, whereas the NS-HC points showed largely higher

IBI's, greater variability and were more scattered. Each square dot is the centroid for the AS-LC (closed) and NS-HC (open) conditions, and the ellipses summarize the pooled within-group scatter for each test condition. The Hotelling T^2 shows that the centroid of the mean and standard deviation under NS-HC and AS-LC conditions were significantly different ($p< 0.001$). This graph illustrates that air stimulation and concentrating on a visual task produce almost opposing effects on the IBI and its variability.

3.2. Blink Amplitude

The average blink amplitude was similar among conditions (Table 1), but there were some individual differences among tasks (Figure 3B). Some subjects showed decreased amplitude and increased variability when concentrating on the computer task (NS-HC), but there was no significant concentration, air stimulus main effects or interactions ($p>0.05$).

Figure 4B compares the mean and standard deviation of the NS-HC and AS_LC and shows that there was extensive overlap between the points, suggesting neither concentration nor the air stimulus markedly effected blink amplitude. The Hotelling T^2 confirms this, demonstrating that the centroid of the mean and standard deviation under the NS-HC and AS-LC conditions were not significantly different from each other ($p= 0.843$).

In agreement with previous results,(Himebaugh, Begley et al. 2009) the majority of blinks in this study were incomplete. Blinks with amplitude below 100% ("partial blinks"), comprised 79% of the total number of blinks. However, 71% of partial blinks

fully covered the pupil. Blinks with amplitudes above 100%, appeared to be due to occasional forceful squeezing of the eyes shut during the blink so that the tracking dot excursion exceeded that of the initial calibration for a full blink, thus registering as >100% (see the arrows in Figure 2C). Most (74%) of these blinks were in the subjects with a previous dry eye diagnosis, suggesting that more frequent, forceful blinks with squeezing of the eyelids may occur in dry eye. However, because this investigation involved only a few dry eye subjects, this observation should be tested in future studies.

3.3. Blink Duration

As others have found,(Evinger, Manning et al. 1991; Bologna, Agostino et al. 2009) the average duration of the down phase of the blink (93±33msec) was significantly shorter compared to the up phase (154± 48msec, paired t-test, $p<0.001$). Regardless of air stimulus, down phase, up phase and total blink duration were significantly shorter during HC tasks compared to LC tasks ($p= 0.010$. 0.044 and 0.013, respectively). No significant air stimulus main effect was found. There were no interactions between task concentration and air stimulus effects.

As with IBI, the variability varied among conditions, in addition to the mean (Figure 4C). In this case, the down phase blink duration with air stimulus (AS-LC) was greater and more variable than with concentration on a task (NS-HC). The Hotelling T^2 shows that the centroid of the mean and standard deviation under the NS-HC and AS-LC were significantly different ($p= 0.0016$), with few points in each group overlapping.

These data suggest that concentrating on a visual task is associated with short duration blinks, whereas air stimulation tends to be associated with blinks of longer duration and higher variability.

3.4. Maximum Velocity

As Table 1 shows, the down phase maximum velocity was much higher than that in the up phase, which agrees with previous reports.(Evinger, Manning et al. 1991; Guitton, Simard et al. 1991; Sforza, Rango et al. 2008) When conditions were compared, there were no concentration or air stimulus main effect on down or up phase velocity, but there was a significant interaction between concentration and air stimulus for down phase maximum velocity ($p=0.021$). During the high concentration tasks, the down phase maximum velocity increased with the air stimulus, while the opposite occurred with the low concentration tasks, suggesting that the change in maximum velocity depends on both attention and the air stimulus.

3.5. Correlation between Blink Amplitude and Maximum Velocity

As in previous studies, there was significant correlation between blink amplitude and maximum velocity when all the data was pooled together ($r= 0.64$ $p<0.001$), suggesting that fuller blinks tend to have a higher maximum velocity.(Evinger, Manning et al. 1991; Guitton, Simard et al. 1991) However, individual subjects varied in the slope and degree of correlation between these parameters. To illustrate this point, Figure 5 shows the relationship between blink amplitude and maximum velocity for all subjects in

this study under all conditions. We divided subjects by previous dry eye diagnosis because we noted that dry eye subjects showed a much greater individual variation in this relationship, compared to subjects who had not previously been diagnosed with dry eye. Pooling all the conditions together, the mean slopes ± standard deviation for non-dry eye and previously diagnosed dry eye subjects were 0.15±0.06 and 0.22±0.15, respectively. Although not conclusive in this pilot study, these data suggest that individual correlations should be examined, rather than the common practice of pooling data from multiple subjects, because dry eye or other subjects may show differing relationships among common blink parameters.

3.6. Tear Film Instability and Its Correlation with Blinking

Table 2 shows the average TBU area before and after blink under the four study conditions. The amount of TBU was significantly decreased after blinking compared to before for all conditions ($p = 0.001$). However, it should be noted that significant TBU often remained after the blink, especially inferiorly. This appeared related to the high number of incomplete blinks (see the section on Blink Amplitude) that failed to fully wet the cornea.

The high variation of TBU before the blink during NS-LC was due to one subject who only blinked five times and who had high TBU area before each blink. With the data from this outlier removed, the TBU average and standard deviation for NS-LC was

5.1±4.5%. There was no statistically significant difference in the percent TBU among the four conditions studied.

Correlations between blink parameters and TBU before or after the blink were low (r ranges from -0.004 to 0.254).(Freudenthaler, Neuf et al. 2003; Himebaugh, Begley et al. 2009) Figure 6 shows the relationship between one blink parameter, the IBI, and TBU before the blink. Similar results were found when data were separated by condition or individual. Although some blinks followed large amounts of TBU over the cornea, many blinks occurred without TBU. In addition, many blinks were incomplete and thus failed to fully clear TBU.

Discussion

This was a pilot study designed to explore the effects of concentration and a relatively mild surface stimulus on multiple blink parameters, which are not often measured on human subjects.(Nakamori, Odawara et al. 1997; Toda, Asano-Kato et al. 2001; Naase, Doughty et al. 2005; Borges, Garcia et al. 2010; Jansen, Begley et al. 2010) We found that an ocular surface air stimulus that was considered barely noticeable produced an increased frequency and regularity of blinking, whereas concentrating on a visual task produced less frequent and more irregularly spaced blinks. Blink duration was decreased when concentrating, presumably to minimize distraction by the eyelid during the task. These data support the hypothesis that blink parameters, including frequency and duration, are modulated by even mild surface input and cognitive

levels.(Nakamori, Odawara et al. 1997; Evinger, Bao et al. 2002; Himebaugh, Begley et al. 2009; Jansen, Begley et al. 2010; Cardona, Garcia et al. 2011)

Blink frequency, as measured by the IBI in this study, increased even with the very mild air stimulus used in this study. Similar results have been found with other presumed ocular surface stimuli, such as wearing contact lenses(Jansen, Begley et al. 2010) or having dry eye.(Nakamori, Odawara et al. 1997; Himebaugh, Begley et al. 2009) The presumed explanation is that ocular surface input, which is in the form of an inadequate tear film in dry eye, tear film evaporation from air stimulus, or a contact lens, acts to stimulate the ocular surface, resulting in increased blinking. While it is difficult to compare the stimuli used in this and previous studies to each other, these results suggest that a wide range of ocular surface inputs, from the barely noticeable air stimulus used in this study to severe dry eye affect blinking, although the degree may vary widely.(Nakamori, Odawara et al. 1997; Naase, Doughty et al. 2005; Himebaugh, Begley et al. 2009; Jansen, Begley et al. 2010)

The variability of the IBI was also affected by the air stimulus used in this study. As the rate of blinking increased with the very mild air stimulus, so did the regularity of blinking.(Tsubota, Hata et al. 1996) Similar results were found in animal models, where stimulation of the ocular surface or supraorbital nerve were associated with an increased spontaneous blink rate and enhanced regularity of the blink pattern.(Kaminer, Powers et al. 2011) Irritation of the ocular surface is known to produce trigeminal reflex blink excitability and extra blinks, termed blink oscillations, that occur at a relatively constant interval after an initial reflex blink.(Evinger, Bao et al. 2002) Blink oscillations occur in

dry eye animal models, leading to the hypothesis that increased blink rate and regularity in dry eye may be an adaptive modulation of the blink response to provide a better tear film.(Evinger, Bao et al. 2002; Kaminer, Powers et al. 2011)

While the air stimulus used in this study increased the rate and regularity of blinking, concentration on a visual task produced the opposite effect (Figures 4A and C). Many have shown a decreased blink rate with concentration and increased attentional state,(Tsubota and Nakamori 1993; Doughty 2001; Freudenthaler, Neuf et al. 2003; Schlote, Kadner et al. 2004) but the temporal distribution also becomes more irregular and tends to cluster(Jansen, Begley et al. 2010) during short-term cognitive encoding of information. In a study of spontaneous blink rates in patients with vegetative conditions, the irregularity of blinking increased with clinical improvement, suggesting that irregular blinking is associated with recovery of consciousness and cognitive thought processing.(Bonfiglio, Carboncini et al. 2005) In this study, subjects playing a computer game and showed a wide variability in both blink rate and regularity (Figures 1, 2 and 3), which may, in part, reflect the degree of attention each subject paid to the game. We did not measure the degree of attention to the visual task in this study, which may have introduced some differences in blink response among subjects. However, the decreased rate and regularity of blinking found among many subjects presumably occurred due to increased cognitive activity with some longer periods between blinks, theoretically to increase information processing.(Cardona, Garcia et al. 2011)

According to previous studies, concentration reduces blink amplitude as well as decreasing the blink rate,(Jansen, Begley et al. 2010; Cardona, Garcia et al. 2011)

presumably to minimize distraction by the eyelids. In this study, there was no statistically significant reduction in blink amplitude while playing the game, although individual subjects did show a reduction in blink amplitude (Figure 1B). This discrepancy might be due to the small sample size in the current study and the different experimental design. In addition, subjects held one eye closed during the study, which may have interfered with the blink response in the tested eye. Regardless of differences in blink amplitude among subjects and conditions, many blinks (79%) were partial in this study. However, even though incomplete blinks were common, 71% of blinks covered more than two thirds of the corneal surface, so that most covered the pupil, providing good tear film over the pupil area. These results agree with a previous study from our laboratory(Himebaugh, Begley et al. 2009; Jansen, Begley et al. 2010) raising questions as to why partial blinking is so common. It is possible that the primary purpose of the blink is to spread the tear film over the pupil to ensure a good optical surface for the eye. This could explain why partial blinks and the resulting inferior tear break-up are so commonly found in this and our previous study.(Himebaugh, Begley et al. 2009) In addition, we have also previously shown that the tear film was more stable among dry eye subjects following a partial compared to a full blink.(Harrison, Begley et al. 2008) Thus, although incomplete blinks are often considered undesirable, their relative frequency raises questions about their contributions to vision, attentional state and tear film stability.

Blink duration also appeared to be affected by attentional state and ocular surface input in this study. The high concentration task produced significantly shorter duration blinks with less variability than the air surface stimulus (Figure 4C), presumably for the

purpose of minimizing interruption by the eyelid during the task. Even in the combined condition of air stimulation and concentration, blink duration was still short and regular (Figure 3C). Blink duration was longer and much more variable with the air stimulus, suggesting that some longer blinks occurred to protect the ocular surface from irritation. Although few have studied spontaneous blink duration under different conditions, increased blink duration is associated with trigeminal excitability, which may occur in response to supraorbital nerve stimulation(Evinger, Manning et al. 1991; VanderWerf, Brassinga et al. 2003) or decreased dopamine levels.(Peshori, Schicatano et al. 2001; Agostino, Bologna et al. 2008)

Others have shown a tight correlation between amplitude and velocity, suggesting that, as the blink becomes fuller, it is faster.(Evinger, Manning et al. 1991) We found a similar result when all data was pooled, as in many previous studies. However, this association was more variable when individual subjects were examined, especially in some subjects who had been previously diagnosed with dry eye (Figure 5). As the slope of this relation is considered to be an indicator of motor neuron activity of eyelid muscles,(Hasan, Baker et al. 1997) this suggests blinking kinetics in individual subjects may differ, perhaps relating to altered ocular surface inputs in dry eye. Some studies have shown that dry eye subjects have desensitized ocular surfaces,(Bourcier, Acosta et al. 2005) although others have shown hypersensitivity in dry eye subjects.(De Paiva and Pflugfelder 2004; Situ, Simpson et al. 2008) While this study was small, exploratory and involved only a few subjects, it may be useful to further examine individual correlations between blink parameters in future studies, rather than pooling data.

Some have suggested that the ocular surface controls blinking because stimulating the surface or a poor tear film in dry eye increases the blink rate, while anesthetic decreases the blink rate.(Nakamori, Odawara et al. 1997) In this and a previous study, we examined the relationship between tear film break-up and blinking, reasoning that an unstable tear film with break-up should stimulate increased blinking. In both studies, tear break-up occurred while subjects played the video game, especially inferiorly, and often was not fully cleared by the frequent partial blinks. However, we were unable to find a direct relationship between tear break-up and blinking in either study.(Himebaugh, Begley et al. 2009) One possibility may be that we measured only tear break-up and not significant thinning. Both are likely to occur in an unstable tear film,(Begley, Simpson et al. 2013) producing transient increased tear hyperosmolarity, which would be likely to stimulate surface neurons.(Liu, Begley et al. 2009) Another possibility is that mild corneal irritation stimulates the trigeminal to increase blinking,(Evinger, Bao et al. 2002; Kaminer, Powers et al. 2011) thus masking the initial stimulus. In addition, we directed the air flow to stimulate the ocular surface, but stimulation may also have occurred to the eyelids during blinking. The air flow may also have increased tear film evaporation which could increase cooling or tear film osmolarity, both of which would stimulate ocular surface neurons.(Acosta, Tan et al. 2001; Belmonte, Acosta et al. 2004) Thus, the nature of the air stimulus and the ocular surface controls over blinking remain unclear.

This study evaluated multiple blink parameters to compare the effects of concentration on a visual task and an air stimulus that was designed to be barely noticeable by subjects. It was an exploratory study designed to raise questions to be addressed in future studies. Even with a small sample size and using subjects with a

range of dry eye symptoms, we found that even a mild air stimulus produced opposing effects on blink frequency and duration when compared to concentration on a task. Although we did not design the study to compare dry eye to normal subjects, the subjects in this study with a previous dry eye diagnosis tended to show more variable blinking kinematics compared to non-dry eye. These results suggest that individual subjects may respond differently, so that future studies are needed to address the ocular surface versus cognitive controls over blinking and their importance in the dry eye condition.

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Table 1: Averages ± standard deviations for all blink parameters under different conditions.

				Blink Duration (msec)			Maximum velocity (mm/sec)	
Concentration	Air stimulus	IBI (sec)	Blink amplitude (%)	Down phase	Up phase	Total	Down phase	Up phase
Low	No (NS-LC)	5±3	86±17	93±23	155±23	260±50	168±61	92±26
	Yes (AS-LC)	3±1	80±18	100±24	169±54	286±80	155±75	88±33
High	No (NS-HC)	6±3	75±23	82±13	142±30	241±33	140±57	83±33
	Yes (AS-HC)	5±3	80±20	81±17	138±26	226±44	161±58	91±23

Gray shading: Significant main effect, HC versus LC, $p<0.05$, repeated measures ANOVA

Table 2: The percent area of TBU before and after the blink (mean± standard deviation) under different conditions.

Condition	Before blink (%)	After blink (%)
NS-LC	8.3±10.6**	2.3±2.2
NS-HC	5.9±4.7**	2.9±2.5
AS-LC	5.6±4.8**	3.3±2.6
AS-HC	4.2±3.4**	2.5±2.1

** significant difference between before and after the blink (paired t test, p=0.001)

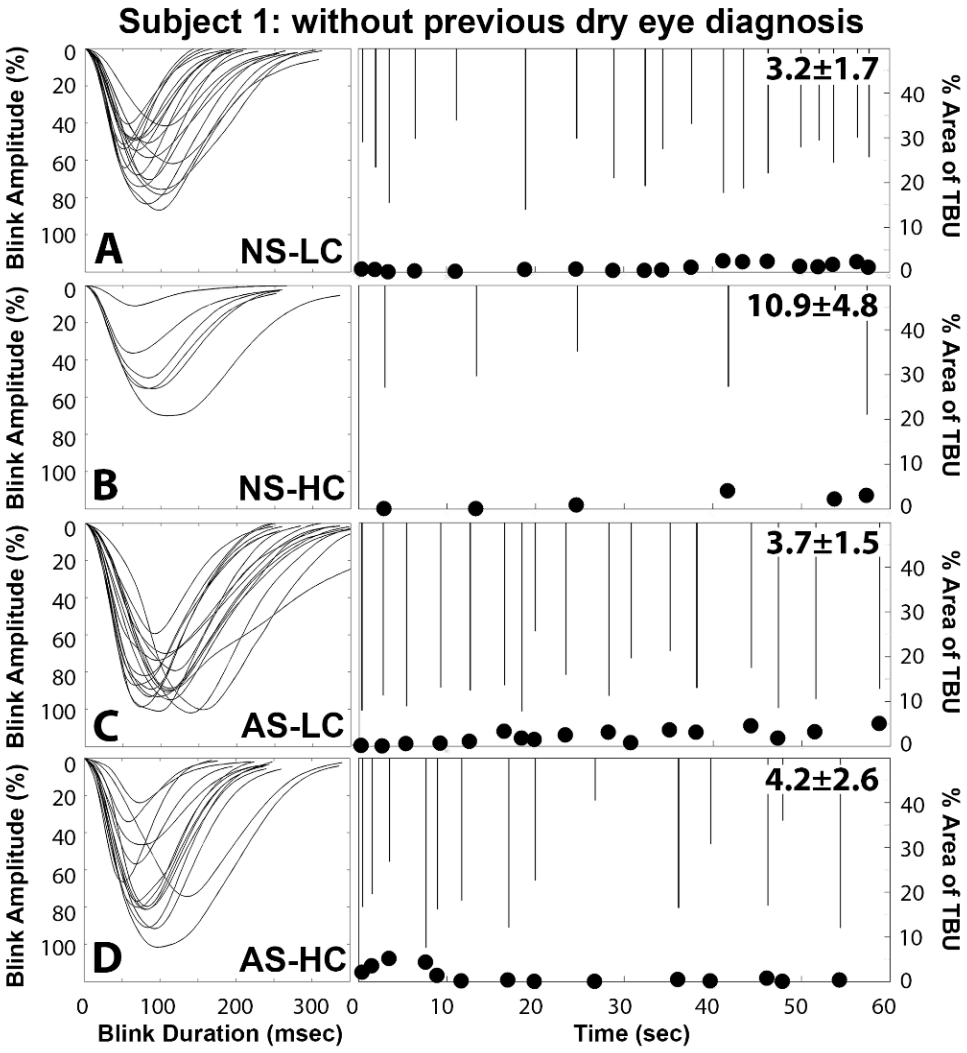

Figure 1: Blink traces (left), blink timing, amplitude (vertical bars), the area of TBU before blink (dots) and the average IBI ± standard deviation during 60 sec trial (right) for Subject 1 under four experimental conditions (A-D). Blink amplitude is displayed on a reverse axis to mimic the direction of the lid during the blink.

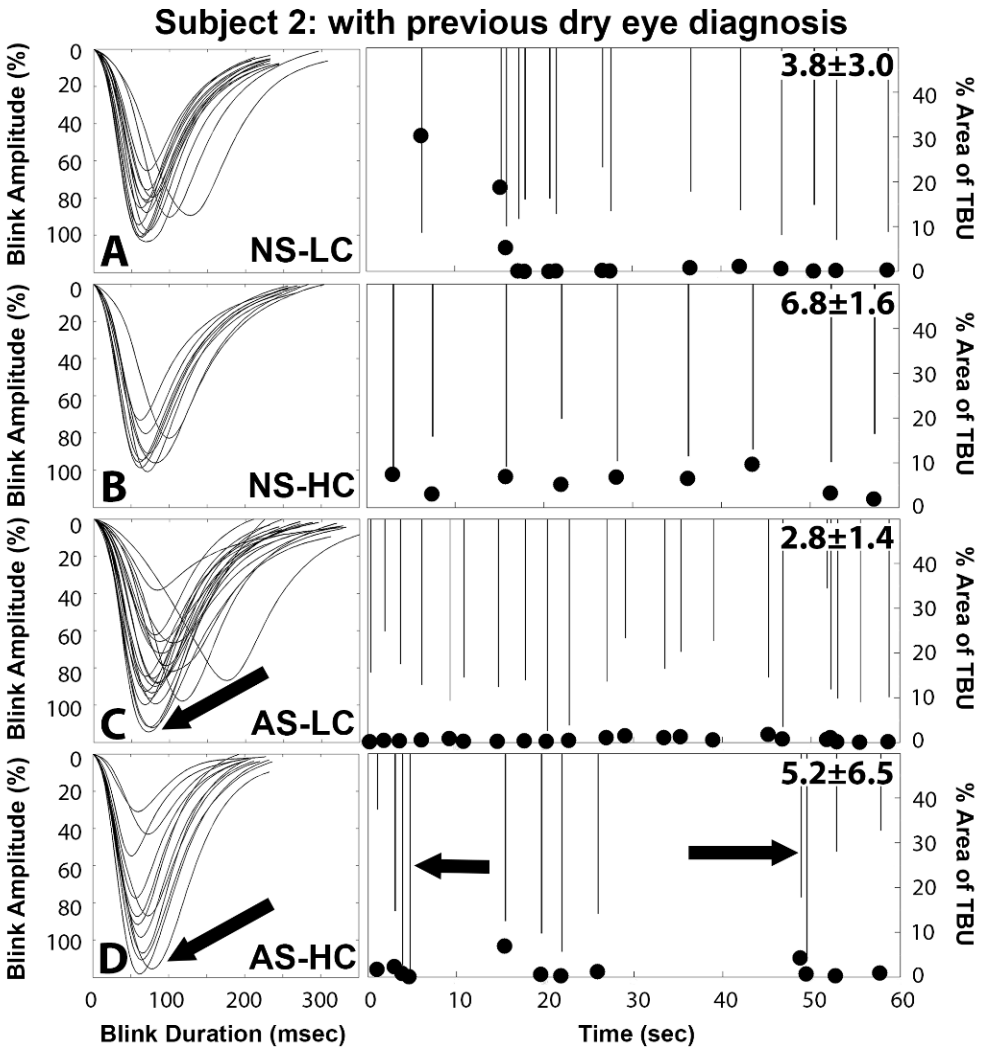

Figure 2: Blink traces (left), blink timing, amplitude (vertical bars), the area of TBU before blink (dots) and the average IBI ± standard deviation during 60 sec trial (right) for Subject 2 under four experimental conditions (A-D). Blink amplitude is displayed on a reverse axis to mimic the direction of the lid during the blink. Open arrow points to the blink amplitude over 100%; filled arrows point to cluster blinks that blinks occurs with short intervals

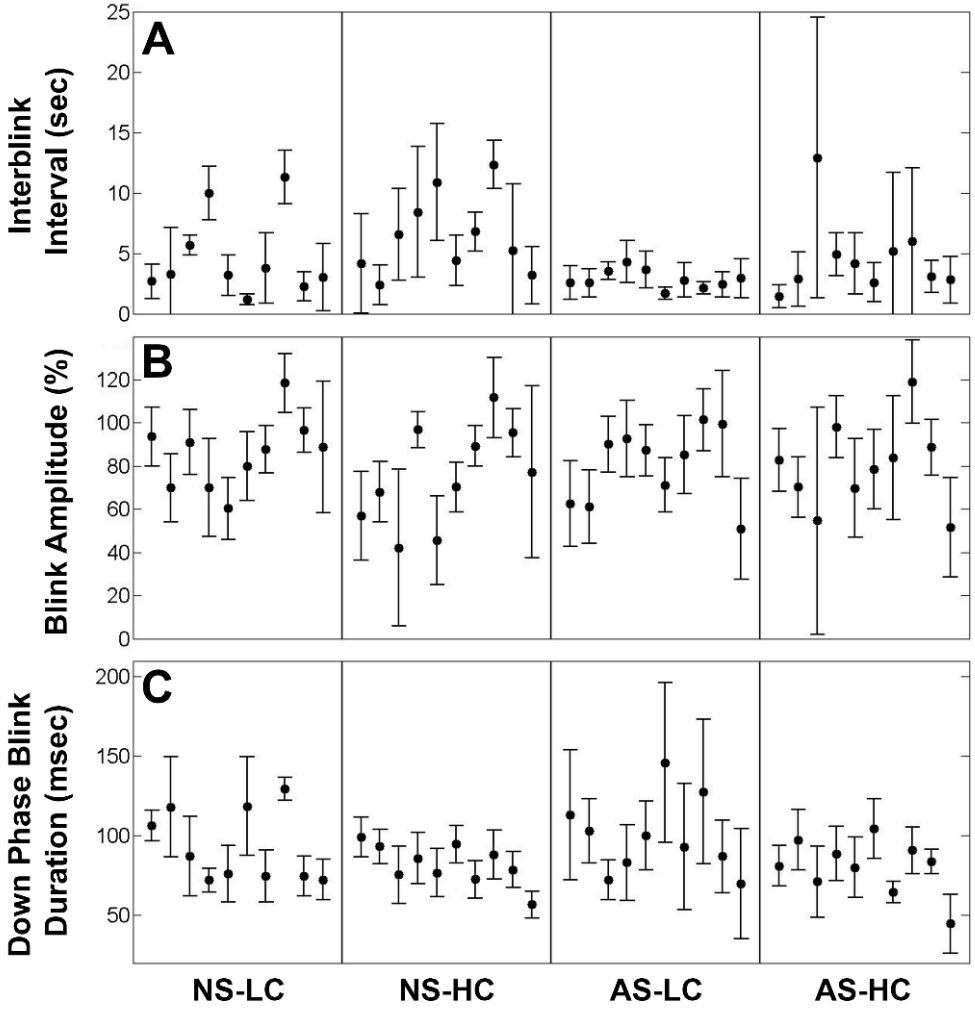

Figure 3: Error plots (showing mean and standard deviation) of the IBI (A), blink amplitude (B), and down phase duration (C) for all subjects under four test conditions.

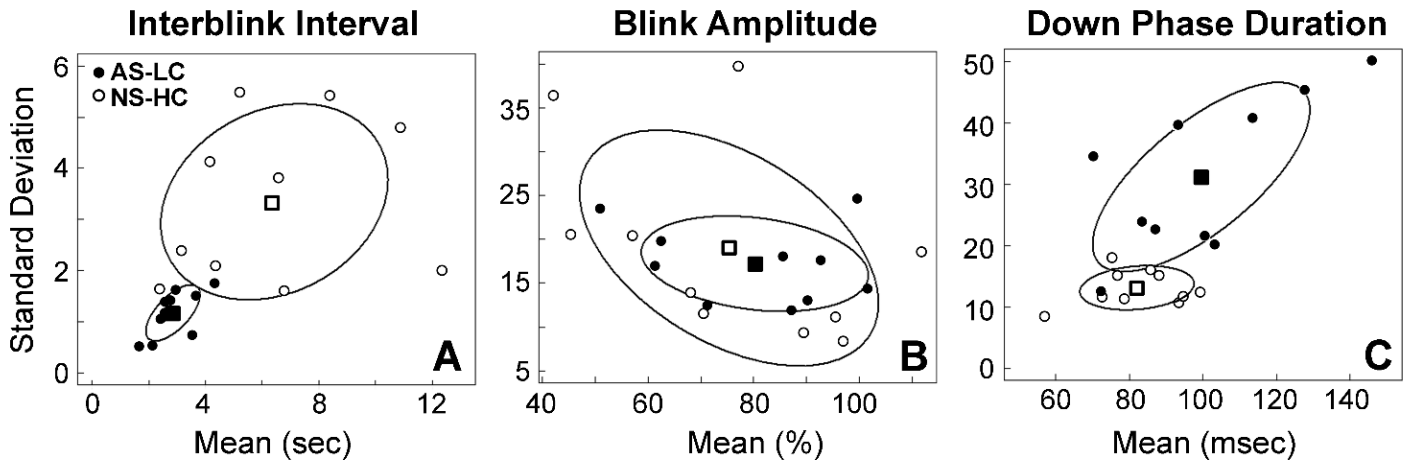

Figure 4: Scatter plots of mean versus the standard deviation of the NS-HC (open circles) and AS-HC (filled circles) of the IBI (A), blink amplitude (B), and down phase duration (C) for all the subjects. The square dots are the centroids for each condition and the ellipses summarize the pooled within-group scatter for each test condition.

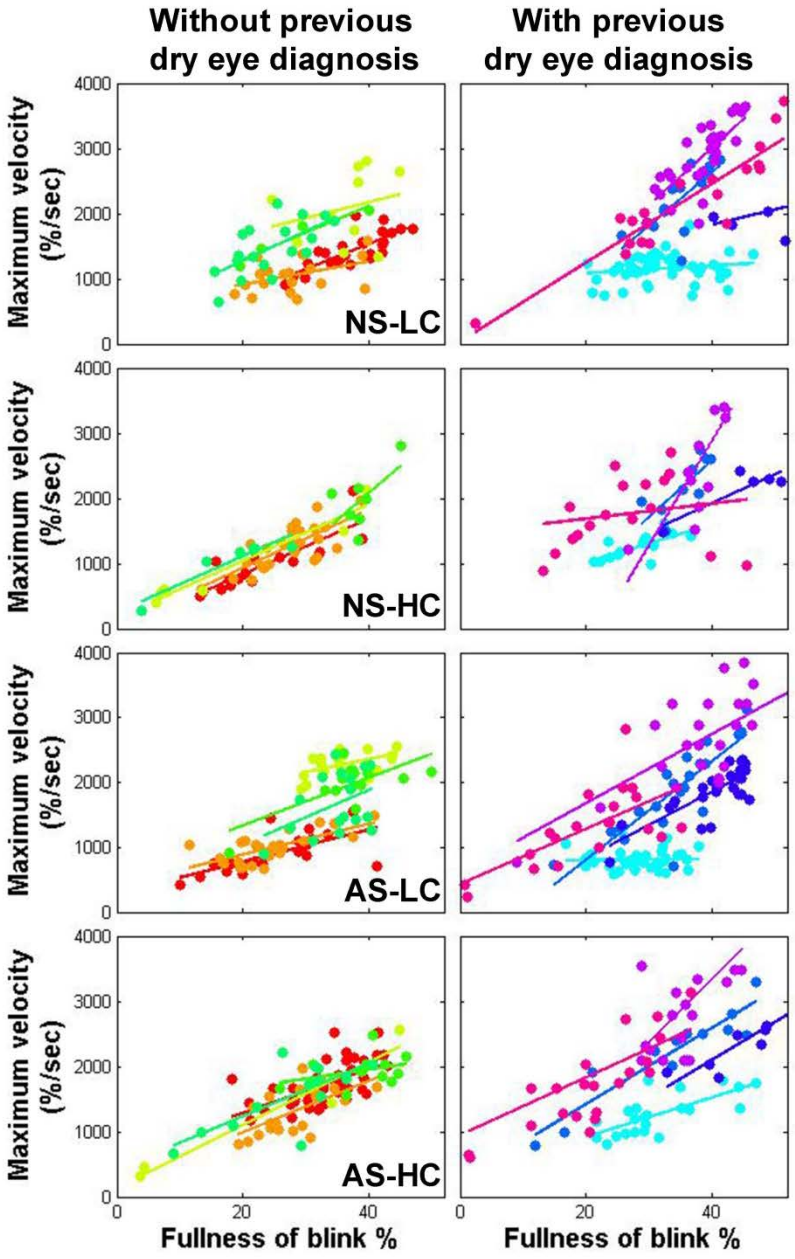

Figure 5: Blink amplitude versus maximum down phase velocity under four conditions for subjects with and without a previous diagnosis of dry eye. Different colors represent different subjects.

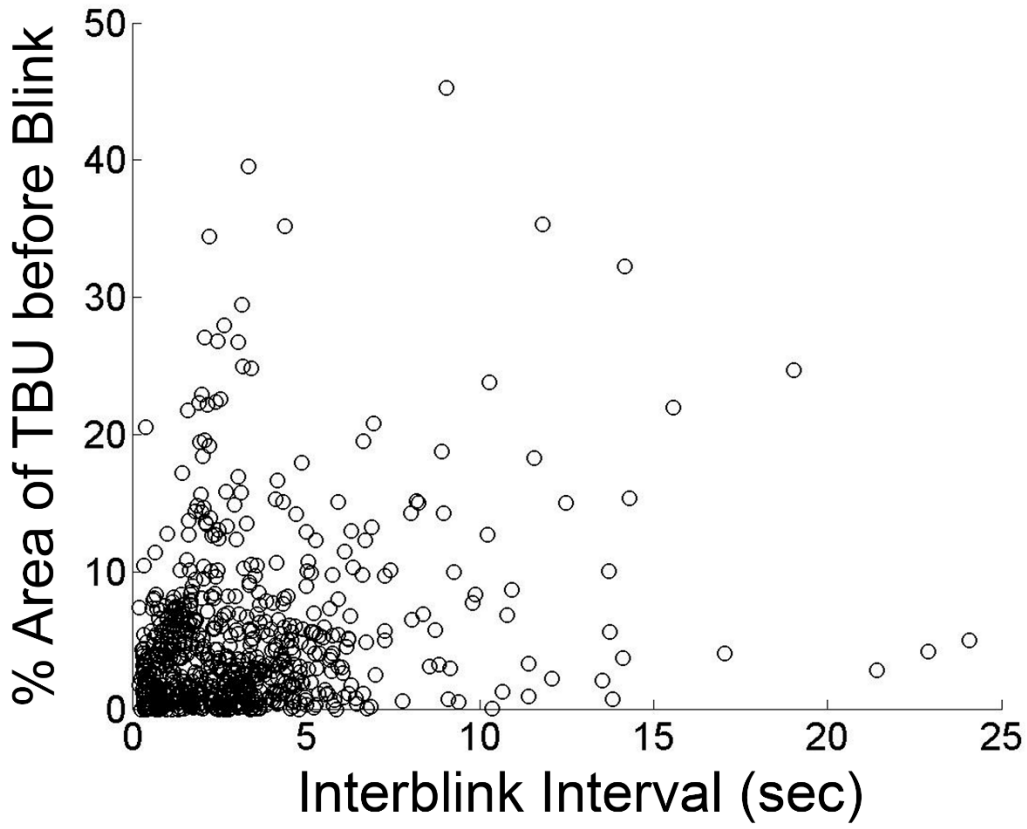

Figure 6: Scatter plots of IBI and area of TBU before the blink for all subjects under all conditions.

CHAPTER III

THE EFFECTS OF INCREASING OCULAR SURFACE STIMULATION ON BLINKING AND SENSATION

Abstract

Purpose: The purpose of this study was to determine how increasing ocular surface stimulation affected blinking and sensation, while controlling task concentration.

Methods: Ten healthy subjects concentrated on a task, while a custom pneumatic device generated air flow toward the central cornea. Six flow rates (FR) were randomly presented 3 times each and subjects used visual analog scales to record their sensory responses. The interblink interval (IBI) and the FR were recorded simultaneously and the IBI, sensory response and corresponding FR were determined for each trial. The FR associated with a statistically significant decrease in IBI, the blink increase threshold (BIT), was calculated for each subject.

Results: Both the mean and standard deviation of IBI were decreased with increasing stimulation, from 5.69±3.96 seconds at baseline to 1.02±0.37 seconds at maximum stimulation. The average BIT was 129±20ml/min flow rate with an IBI of 2.33±1.10 seconds (permutation test, $p<0.001$). After log transformation, there was a significant linear correlation between increasing FR and decreasing IBI within each subject

(Pearson's $r \leq -0.859$, $p<0.05$). The IBI was highly correlated with watery, discomfort and cooling ratings (Pearson's $r \leq -0.606$, $p<0.001$).

Conclusions: There was a dose-response between increased surface stimulation and blinking in normal subjects, presumably for protection of the ocular surface. The blink response was highly correlated with ocular surface sensation, which is not surprising given their common origins. The BIT (blink increase threshold), a novel metric, may provide an additional endpoint for studies on dry eye or other conditions.

This project has been published in Investigative Ophthalmology and Visual Science. (Wu, Begley et al. 2014)

Introduction

Dry eye affects millions in the US(Schaumberg, Sullivan et al. 2003; Schaumberg, Dana et al. 2009) and worldwide.(McCarty, Bansal et al. 1998; Uchino, Nishiwaki et al. 2011) It is considered to be a multifactorial condition driven by tear film instability and hyperosmolarity,(2007) with reduced blink rate as a potential risk factor that may exacerbate the condition through increased tear film evaporation.(Tsubota and Nakamori 1995; 2007) The blink acts to spread the tear film and rewet the ocular surface,(Doane 1980; Oyster 1999; Palakuru, Wang et al. 2007) so that the quantity and quality of the tear film may also be affected by the blink.(Harrison, Begley et al. 2008; Hirota, Uozato et al. 2013) However, despite its importance for ocular surface wetting and the dry eye condition, the ocular surface controls over blinking remain controversial.(Karson 1983; Nakamori, Odawara et al. 1997; Doughty and Naase 2006; Kaminer, Powers et al. 2011)

Previous studies have shown that the blink rate (BR) or inter-blink interval (IBI) is affected by central dopamine level,(Karson 1983; Taylor, Elsworth et al. 1999) cognitive state(Patel, Henderson et al. 1991; Doughty 2001) and ocular surface input.(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Naase, Doughty et al. 2005; Wu, Begley et al. 2013) Reading, working on computer, or other visual tasks requiring concentration are known to decrease blink frequency,(Acosta, Gallar et al. 1999; Schlote, Kadner et al. 2004; Himebaugh, Begley et al. 2009; Cardona, Garcia et al. 2011) whereas irritation or stimulation of the ocular surface increases the BR.(Tsubota, Hata et al. 1996; Nakamori, Odawara et al. 1997; Wu, Begley et al. 2013) The dry eye

condition is associated with an increased BR,(Tsubota, Hata et al. 1996; Himebaugh, Begley et al. 2009) presumably due the ocular surface irritation and stress provided by an unstable or hyperosmolar tear film.(Liu, Begley et al. 2009; Begley, Simpson et al. 2013) Likewise, air blowing to the ocular surface, which induces mechanical force onto and evaporation of the tear film, has been shown to increase BR under a variety of experimental conditions and in subjects with and without dry eye.(Tsubota, Hata et al. 1996; Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Wu, Begley et al. 2013) Pneumatic stimulation provides the possibility of a controlled, laboratory based method to further study the effect of ocular surface stimulation on blinking.

The neural pathways involved in ocular surface controls over reflex blinking arise in ocular surface sensory nerves that project to the motor neuron of seventh cranial nerve through trigeminal sensory fibers.(Hiraoka and Shimamura 1977; Pellegrini, Horn et al. 1995) Recently, Kaminer et al. hypothesized that the spinal trigeminal complex plays an important role in generating the spontaneous blink by modulating direct signals from ocular surface and indirect signals from the basal ganglia to vary the blink pattern.(Kaminer, Powers et al. 2011) Furthermore, stimulation of the ocular surface by the dry eye condition, in both humans and animal models, increased both BR and the regularity of blinking.(Peshori, Schicatano et al. 2001; Evinger, Bao et al. 2002) Thus, ocular surface afferent input appeared to be responsible for increasing blink frequency and regularity.(Wu, Begley et al. 2013) However, the effect of varying the level of ocular surface stimulation on BR has not been explored under controlled experimental conditions.

Sensations of eye discomfort also begin with stimulation of ocular surface afferent sensory neurons, although the reflex blink and sensory pathways diverge at the level of the spinal trigeminal complex.(Stapleton, Marfurt et al. 2013) While the changes in the BR(Tsubota, Hata et al. 1996; Nakamori, Odawara et al. 1997; Himebaugh, Begley et al. 2009) and symptoms of ocular irritation(Schein, Tielsch et al. 1997; Nichols, Begley et al. 1999; Begley, Caffery et al. 2002) are both associated with the dry eye condition, few studies have used human subjects to systematically examine their relationship to each other, despite common origins. Therefore, in this study, we employed a human-based laboratory model to investigate the effect of varying levels of pneumatic stimulation of the ocular surface on blinking and ocular sensation. During the study, all subjects were engaged in a visual task to minimize the effect of variations in concentration on blinking to better isolate the effect of ocular surface stimulation. Only young, healthy subjects were included in this study to determine the responses within a normal physiological range (i.e., healthy ocular surface and asymptomatic), thus avoiding the potential variability that putative sensory and other nerve damage associated with dry eye or other conditions could add.(Bourcier, Acosta et al. 2005; De Paiva, Chen et al. 2006; Toda, Kato-Asano et al. 2006; Situ, Simpson et al. 2008; Tuisku, Konttinen et al. 2008)

Methods

1. Subjects

The study was conducted at the Borish Center for Ophthalmic Research at the Indiana University School of Optometry, Bloomington, Indiana. It adhered to the tenets

of the Declaration of Helsinki and was approved by the Institutional Review Board at Indiana University. Informed consent was obtained from each subject prior to beginning the study.

Ten young, healthy subjects were recruited for the study. Subjects reporting ophthalmic disorders, including dry eye, ocular or systemic allergies, any systemic disease or contact lens wear were excluded.

All subject visits were scheduled for approximately the same time of the day (between 1:00 and 1:30pm).(Barbato, Ficca et al. 2000) At the beginning of the study, subjects were told that the reason for the study was to examine the tear film while they were engaged a computer task. They were *not* informed that the purpose included monitoring of blinking until the study was completed to avoid any potential cognitive or affective contaminating effects on blinking.(Doane 1980)

2. Experimental Procedures

There was one visit in this study. After filling out the Dry Eye Questionnaire (DEQ)(Begley, Caffery et al. 2002) to assess habitual symptoms of ocular irritation and dry eye, subjects were seated behind a slit lamp biomicroscope (Zeiss 20SL, Carl Zeiss, Germany, 8x magnification) with a custom-attached camera (Basler piA640-210gm, Basler AG, Germany, 30Hz), recording the movement of the upper lid. In order to quantify eyelid movement, a self-adhesive 2 mm diameter reflective white dot (3M Company, Minnesota, USA) was gently positioned as close as possible to the margin of the right upper lid. Subjects looked straight ahead and played a computer game (Tetris®)

viewed through a beam splitter. Only the right eye was tested. The other eye was manually held shut by the subject.

An instrument similar to a pneumatic esthesiometer was used to stimulate the cornea with air flow.(Belmonte, Acosta et al. 1999; Situ, Simpson et al. 2008) It consisted essentially of an air pump (using atmospheric air), a voltage regulated valve to control the flow rate (FR), an approximately 1 liter reservoir to minimize the slight irregularities in flow from the pump, and a sensor measuring the actual flow.(Vega, Simpson et al. 1999) Air was delivered through a hypodermic syringe with a 0.5mm diameter mounted on a slit lamp biomicroscope. The air stimulus was aimed toward the center of cornea, but at a slight angle (12 degrees from horizontal and 5 degrees from vertical) so that it did not block the slit lamp view of the cornea or the subject's vision while playing the computer game. The distance between the tip (metal) of the air stimulus and the cornea was 15mm and its position was constantly monitored by a calibrated side mounted camera. The FR from the stimulus tip was recorded by through a customized LabVIEW 5.1 program and time stamps were used to relate stimulus timing with blink data.

In order to estimate the level of pneumatic stimulus that triggered a higher BR, the stimulus FR was systematically increased from zero every 30 seconds in a step size of 50ml/min. The experimenter initially (by simple observation) estimated the level that appeared to produce consistently increased BR. After 5 minutes this procedure was repeated and a final estimate obtained from the average of the two trials. This estimate was then used to set the six levels of pneumatic stimuli to be tested for each individual subject in the study by multiplying this initial estimate with 0, 0.25, 0.5, 0.75, 1, and

1.25; thereby estimating a range of sub- to supra-threshold stimuli producing an increased BR for each subject.

A randomly ordered presentation of these 6 FR was used to determine the effect of each level of stimulation on the IBI for each subject. Each trial began with no stimulus for one minute, continued with a stimulus for two minutes and was followed another minute without the stimulus (Figure 1 and 3). Three sets of randomly presented stimuli (six levels, three repeats, total 18 trials) were applied with at least one minute break between trials. During all testing, overhead lights in the testing room were turned off and infrared light was used to image the lid, in an attempt to avoid reflex blinking and tearing from visible light. The subject was asked to blink twice before each trial began. Immediately after each trial, using visual analog scales (VAS), subjects rated specific ocular sensations (cooling, wateriness, discomfort, burning and dryness) that they experienced during the stimulus period. Each VAS consisted of a continuous line with zero labeled as no sensation and 10 labeled as the severe sensation. Subjects viewed the VAS on a computer screen and used a mouse to position the cursor on the line to quantify their experience. At the end of the study, a Schirmer's I tear test (without anesthetic) and a fluorescein tear break up time (TBUT) test were performed.

3. Blink Analysis

A custom MATLAB® (The Mathworks™, Natick, MA) program was used to track the Purkinje I image,(Freudenthaler, Neuf et al. 2003) located approximately at the center of the cornea. A blink was registered if the Purkinje I image was covered by the

eyelid. All the detected blinks, including full and partial blinks that covered the Purkinje image, were treated as identical event markers to measure the temporal pattern of blinks.(Kaminer, Powers et al. 2011) During each trial, the blink activity and FR were overlaid using time stamps and the IBI was calculated.

4. Statistical Analysis

The IBI data from the three repeated sets were pooled due to the variability inherent in the blink response.(Kaminer, Powers et al. 2011) Because IBIs are often not normally distributed with unbalanced sample size and are highly variable among subjects,(Doughty 2001) we employed a permutation test(Berger 2000) to determine the FR at which the median of IBI was significantly changed from the baseline for each subject (corrected for multiple comparisons to $p \leqq 0.0033$). We considered this point, the blink increase threshold (BIT), to mark the level at which there was significant blink changed associated with pneumatic stimulation in our model. The Brown-Forsythe test was used to test whether the IBI variability differed between the 6 stimulus levels within each subject.(Brown and Forsythe 1974)

For the sensory VAS data, the three repeated trials were combined for each subject, and the Stevens' Power function(Stevens 1970) was fitted between FR and sensory rating. We did not test for a "threshold" similar to the BIT for sensory data due to its more subjective nature. The Pearson's correlation coefficient was used to determine associations between the flow rate, IBI and sensory ratings.

Results

1. Subject

The average (±SD) age of study subjects was 23.8±3 years (range: 19 - 29 years). Five were female and five were male. The median DEQ-5 score(Chalmers, Begley et al. 2010) was 2.5 (range: 0-14), and none of the subjects reported a previous dry eye diagnosis on the DEQ or thought they had dry eye.(Begley, Chalmers et al. 2003) The average (±SD) Schirmer's I tear test and TBUT were 21.2±11.9 mm/5min (range: 2-41mm) and 7.22±4.55 seconds (range: 2-58 seconds), respectively. The average (± SD) of the temperature and humidity in exam room were 24.2±0.5° C and 28.4±6.7%, respectively.

2. Blink Response to Air Stimulation

Figure 1 shows an individual example (Subject 1) of the blink response to six levels of stimulation. During baseline testing, the IBI was irregular with an average (± SD) of 12.5±6.9 seconds. With increasing stimulation, the IBI and its variability markedly decreased from the 73ml/min to the 357 ml/min air stimulus. Corresponding BRs were 10.5, 19.5, 34, 29 and 40.5blinks/min, respectively. Figure 2A shows a histogram of the IBIs from Subject 1, with pooled the results from the three repeated sets of six stimulus levels. As reported in previous studies, the IBI distribution was asymmetric with a J-shaped positive skew at baseline.(Carney and Hill 1982; Doughty 2002; Borges, Garcia et al. 2010) The distribution becomes less skewed with more short

IBIs and fewer long IBIs while increasing stimulation. Figure 2B shows that both IBI and its variability decreased with increasing stimulation. Log transformation (Figure 2C and D) of IBI data reduces the skew of the data and it appears to be a normal distribution,(Borges, Garcia et al. 2010) thus improving visualization and showing a leftward shift in IBI as stimulation increased.

The decrease in the median of IBIs with FR of 73ml/min was statistically significant compared to the baseline (permutation test, $p<0.001$), and thus represented what we defined as the threshold stimulus intensity to produce a significant change in IBI (BIT) in this case (Figure 1). In addition, the variability of the IBI decreased significantly with increasing stimulation (Brown-Forsythe test, $p<0.001$). Figure 2D shows a significant linear correlation between FR and the mean of log IBI ($r= -0.987$, $p=0.0002$). Log transformation of the data in Figure 2D also demonstrates that, as the IBI decreased with increasing FR, its variability decreased proportionally, making the standard deviations of IBI similar in the log scale for this subject.

Subject 2 in Figure 3 demonstrates a very different initial blinking pattern, much more infrequent and irregular at baseline. The pooled IBI histogram in Figure 4A shows the positive skew of the data at baseline, with some IBIs as long as 67 seconds. With increasing stimulation, the IBI decreased (Figure 4B) and the BIT was 193 ml/min (permutation test, $p<0.001$). The IBI variability also decreased significantly with increasing stimulation (Brown-Forsythe test, $p<0.001$). Log transformation of IBI data (Figure 4C and D) shows a leftward shift and increasingly peaked data with increasing stimulation. In contrast to Subject 1, the variability was less proportional to the

decreasing mean, leading to the more variable standard deviations of the IBI in the log scale (Figure 4D). The IBI and FR were highly correlated ($r= -0.953$, $p=0.0033$).

Figure 5 shows the relationship between IBI and FR for all subjects. The average BIT (± standard error) was 129± 20ml/min, ranging from 65 to 193ml/min with an average IBI of 2.33± 1.10 seconds. Although there was high individual variation of IBI during baseline (IBI= 5.69± 3.96 seconds), the IBI decreased to 1.02±0.37 seconds at maximum FR, resulting in similar, short IBIs. The variability of the IBI significantly decreased with increasing surface stimulation within all subjects ($p<0.001$, Brown-Forsythe test). After log transformation (Figure 5B), significant linear functions were fitted to each subject's data, with an average (± SD) slope of -0.0023± 0.0006 and Pearson's r values ranging from -0.859 to -0.988 ($p<0.05$).

3. Sensory Response to Air Stimulation

Figure 6 shows the VAS scores for watery, discomfort, cooling, burning and dryness at different FR for Subjects 1 and 2. Each data point represents an average and standard deviations from the three repeated trials. Subject 1 (Figure 6A) showed the greatest response for watery and discomfort, whereas Subject 2 (Figure 6B) reported mostly wateriness. Other subjects (data not shown) showed similarly increasing ratings for each of the sensory attributes with increasing stimulation.

The Stevens' Power functions were fit to the average sensory data for each subject from the three repeated trials.(Stevens 1970) Individual subject data for watery, discomfort and cooling are shown in Figure 7. In Figure 7A, the watery response for nine

subjects increased with increasing air stimulation, but one subject showed no response (closed arrow). Discomfort (Figure 7B) was similar, but typically lower for many subjects and cooling (Figure 7C) exhibited more variation. The subject indicated with the arrow in Figure 7A followed a similar response for discomfort and cooling (not indicated with an arrow).

Table 1shows average exponent and constant of Stevens' Power function for nine subjects. One subject (Figure 7A, closed arrow) was excluded due to an overall lack of sensory response to stimulation. As Table 1 shows, r square values were generally high, suggesting a good fit for the other nine subjects with the power function. As expected, there was some individual variation of fitted power functions among subjects. The exponents of these power functions for watery and discomfort ratings were >1, suggesting acceleration of these sensory responses. Cooling, burning and dryness fitted power functions had average exponents≈1, and there was also variation among subjects for these attributes.

4. Correlation between Blink and Sensory Responses

Figure 8 shows the relationship between the log of the IBI and the log of the sensory data for all subjects (data pooled). As Figure 8 illustrates, this was statistically significant for the watery, discomfort and cooling sensations (Pearson's r= -0.737, -0.606 and -0.632 respectively, all $p<0.001$). Burning and dryness were also statistically significant, but the correlations were lower and are not shown (r=-0.470 and -0.466 respectively, both $p<0.001$).

Discussion

The results of this experiment support the hypothesis that ocular surface stimulation increases the blink rate and its regularity, presumably as a protective mechanism.(Peshori, Schicatano et al. 2001; Evinger, Bao et al. 2002; Wu, Begley et al. 2013) When task concentration was controlled, there was a linear relationship between stimulus flow rate and IBI, further suggesting a dose-response relationship between ocular surface input and blinking. The ocular sensory response was highly correlated with the blink response, as might be expected considering that they share the same initial input from the ocular surface.(Stapleton, Marfurt et al. 2013)

Previous studies have found that blinking increased with air stimulation to the ocular surface(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Wu, Begley et al. 2013) and other presumed stimuli, such as wearing contact lenses(Jansen, Begley et al. 2010) or the dry eye condition.(Tsubota, Hata et al. 1996; Nakamori, Odawara et al. 1997; Himebaugh, Begley et al. 2009) While many of these studies showed an effect on blinking, the level of surface stimulation was often difficult to quantify.(Himebaugh, Begley et al. 2009; Jansen, Begley et al. 2010) The effect of concentration on a visual task is especially important as it is known to exert a sizeable inhibitory effect on the blink rate.(Schlote, Kadner et al. 2004; Cardona, Garcia et al. 2011) In this study, we attempted to control task concentration as much as possible while delivering known stimuli at several levels, thus emphasizing the measurable effect of ocular surface stimulation on blinking.

One of the main purposes of this study was to explore the relationship between ocular stimulation and blink response. As previous studies have shown, there was some variations in IBI among healthy subjects when no stimulus was applied (Figure 5A).(Carney and Hill 1982; Doughty 2002) Subjects in this study were engaged in a visual task and showed the typical "J" shaped distribution with some longer IBIs, which might be expected when concentrating on a computer game (Figure 2A and 4B).(Doughty 2002) However, with application of the pneumatic stimulus, the blink response among subjects became increasingly similar, as was the linear slope of the decrease in IBI (Figure 5B). Given that this study involved young healthy subjects, the similar slope of changes in IBI appears to reflect a comparable, and relatively uniform physiological response, perhaps 'designed' to quantify external stimulation and respond with appropriate blinking to protect the ocular surface.(Oyster 1999)

While the normal, young healthy subjects in this study showed linear relationships between ocular surface stimulation and blinking, in those experiencing more stimulation due to pathological dry eye conditions might be expected to produce an altered response, depending on the condition of the ocular surface. Previous studies have shown both increased and decreased sensory thresholds in dry eye, presumably due to damage or injury to sensory neurons.(Bourcier, Acosta et al. 2005; De Paiva, Chen et al. 2006; Toda, Kato-Asano et al. 2006; Situ, Simpson et al. 2008; Tuisku, Konttinen et al. 2008) Thus, an altered blink response to increasing ocular surface stimulation might be expected in dry eye, although not tested in this study.

In this study, we quantified the pneumatic FR that associated with a statistically significant change in the blinking and introduced a new term, blink increase threshold (BIT). Given the linear relationship between IBI and flow rate, this may seem an unnecessary and artificial distinction. However, the purpose is to establish it as an additional endpoint for later experimental manipulation of testing conditions that may be expected to affect BR. Because the inputs for triggering an ocular surface stimulated blink depends on the integrity of surface nerves,(Stapleton, Marfurt et al. 2013) this may be a useful measure for understanding the sensory response in a number of circumstances or in subjects with a number of conditions, such as dry eye. However, since this threshold might be expected to vary among subjects with differing levels of concentration(Cardona, Garcia et al. 2011), this extraneous (or confounding) variable would need to be well controlled when using the blink change threshold as an experimental outcome variable.

In this study, increasing stimulation of the ocular surface affected both the IBI and its variability. As the rate of blinking increased with the stimulation, so did the regularity of blinking, although the increase was proportional to the IBI in some subjects when the results were scaled (compare Figure 2B and 4B). Similarly, in animal and human models, stimulation of the ocular surface or supraorbital nerve was associated with an increased spontaneous blink rate and enhanced regularity of the blink pattern.(Peshori, Schicatano et al. 2001; Evinger, Bao et al. 2002; Kaminer, Powers et al. 2011) Irritation of the ocular surface is suggested to produce trigeminal reflex blink excitability, which is associated with extra blinks at relatively constant intervals after an initial reflex blink, termed blink oscillations.(Peshori, Schicatano et al. 2001) In addition, we(Himebaugh,

Begley et al. 2009; Jansen, Begley et al. 2010; Wu, Begley et al. 2013) and others(Doughty 2002) have noted cluster blinks in dry eye or normal subjects, which may be a similar phenomenon. Both blink oscillations and cluster blinks may transiently increase blink rate and regularity in response to surface stimulation, perhaps as an adaptive modulation of the blink response to provide both ocular surface protection and more rapid tear film renewal.(Peshori, Schicatano et al. 2001; Evinger, Bao et al. 2002)

The sensory response also increased with increasing ocular surface stimulation, but the results were more variable than the IBI among subjects for all sensations tested. Although the initial afferent for blinking and sensation is the same up to the level of the trigeminal ganglion complex, blink and sensory pathways then diverge.(Stapleton, Marfurt et al. 2013) Judgments of sensation involve higher centers in the brain, which are perhaps an additional basis for differing responses among individuals. However, despite these differences in sensory reports among subjects, the correlations between IBI and the sensory ratings were quite high for of watery, discomfort and cooling sensations, underscoring the possibility of a common origin of blinking and the sensory response at the ocular surface.

The sensory input at the level of the ocular surface in this study is likely to be the result stimulation of multiple types of neurons.(Acosta, Tan et al. 2001) The air stimulus was presented at room temperature and thus was likely to stimulate both mechanical and thermal receptors, through surface deformation and cooling.(Murphy, Patel et al. 1996; Golebiowski, Lim et al. 2013) Recent evidence has linked tear secretion with stimulation of cooling receptors,(Belmonte, Aracil et al. 2004; Parra, Madrid et al. 2010) which may

account for the relatively uniform watery response among subjects. Cooling sensations were possibly linked to stimulus air temperature, although tear film evaporation with air flow could also stimulate these 'cold' receptors.(Craig, Singh et al. 2000) More rapid evaporation of the tears could also lead to tear film hyperosmolarity, which may stimulate chemical polymodal nociceptors(Acosta, Tan et al. 2001) and result in the burning sensation reported by some subjects.(Liu, Begley et al. 2009; Begley, Simpson et al. 2013) Discomfort is considered a global sensation and may be due to a mixed input from sensory neurons. The origin of the sensation of dryness is poorly understood and reports of this sensation were variable in this group of subjects. However, although we used a potentially mixed stimulus to gauge the ocular surface-derived blink response, the results were surprisingly similar in this group of normal young subjects. This may be due to the robustness of the protective blink and sensory response, both of which are designed to induce the individual to blink and move quickly away from adverse stimuli.(Oyster 1999)

This study involved several limitations that may have affected our results. We used a custom built device, similar to Belmonte or Murphy's esthesiometer,(Murphy, Patel et al. 1996; Belmonte, Acosta et al. 1999) to stimulate the cornea. However, the pneumatic stimulus could also strike the lids during the blink, which could affect the blink rate. In addition, subjects were asked to hold one eye shut to avoid any stimulation from the non-tested eye, which may have affected the subject's natural blink response. However, all experiments were performed under this condition, so comparisons between trials within a subject should minimize this effect.

The study addressed whether ocular surface stimulation affects blinking, a controversial question important in dry eye research.(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Naase, Doughty et al. 2005; Cardona, Garcia et al. 2011; Kaminer, Powers et al. 2011) Although previous studies have yielded differing results,(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999) we controlled task concentration and demonstrated a dose-response like relationship between ocular surface stimulation and the blink response in normal subjects. In addition, we showed high correlations between the blink response and some ocular surface sensations, which highlights their common origin.(Stapleton, Marfurt et al. 2013) These methods and the novel metric, BIT, hold promise for understanding ocular surface sensory input in dry eye and other related conditions and may provide a basis for connecting sensory data to more objective, measurable outputs such as blinking.

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Table 1: The sensory response to different flow rates fitted with the Stevens' power function. The average parameters for the Stevens' power function (y= a* x^b, a: constant, b: exponent) are shown below.

	a (constant)	**b (exponent)**	**R square**
Watery	0.082± 0.238	1.722± 1.099	0.826
Discomfort	0.075± 0.214	1.303±0.492	0.780
Cooling	0.153± 0.413	0.975± 0.415	0.714
Burning	0.208±0.328	0.929±0.858	0.531
Dry	0.458± 0.617	0.850±0.835	0.587

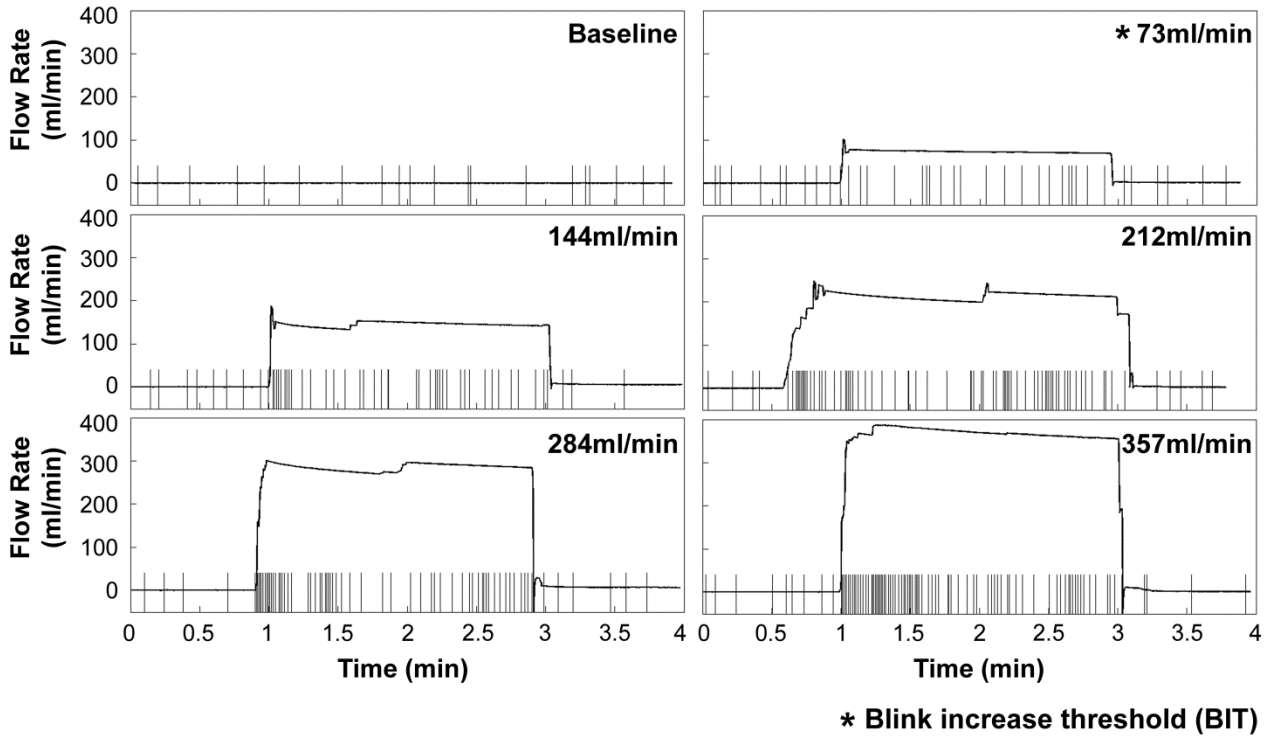

Figure 1: Blink response (Subject 1) to six levels of air stimulation from 0 (Baseline) to 357ml/min. Within each individual graph, the small vertical bars denote the timing of blinks and the horizontal line indicates the flow rate over the trial, with the average flow rate during the central 2 minutes shown at the top right corner. The blink increase threshold (BIT) for this trial was 73ml/min.

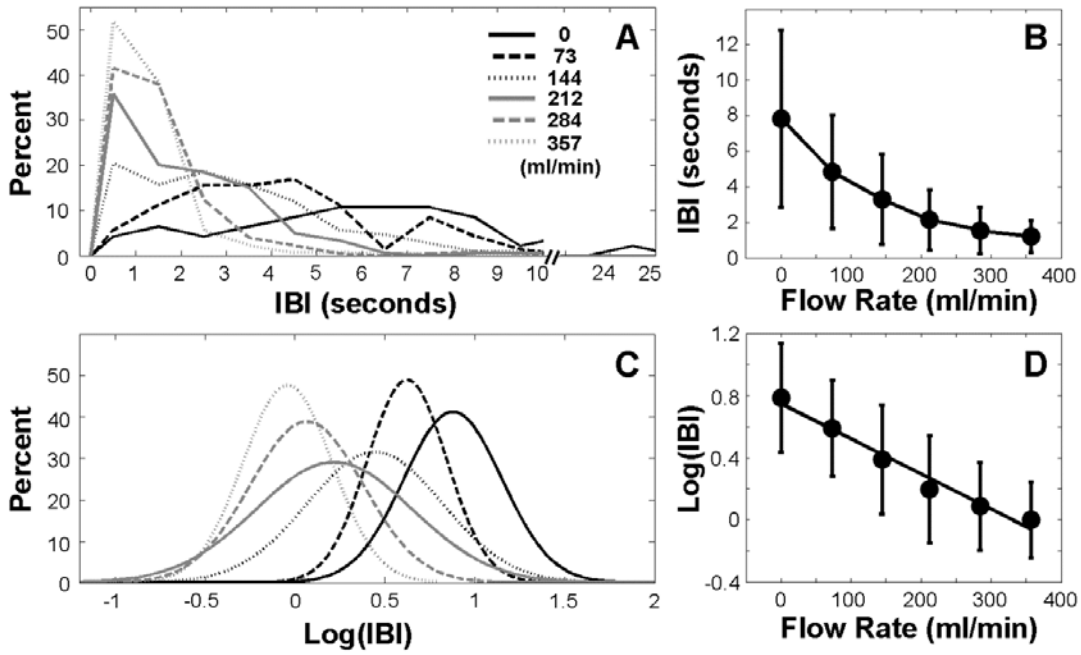

Figure 2: Individual example (Subject 1) using pooled data from three sets of trials. A: IBI distributions under different flow rates. The y axis is the IBI frequency in percent of the total number of IBI data points. B: Mean and standard deviation of IBI as a function of flow rate. C: Log transformation of IBI distributions under different flow rates. Each histogram was fitted with normal curve, and only the best fitted normal curves are shown here. D: Mean and standard deviation of IBI after log transformation as a function of flow rate. A linear regression line was fitted to the data.

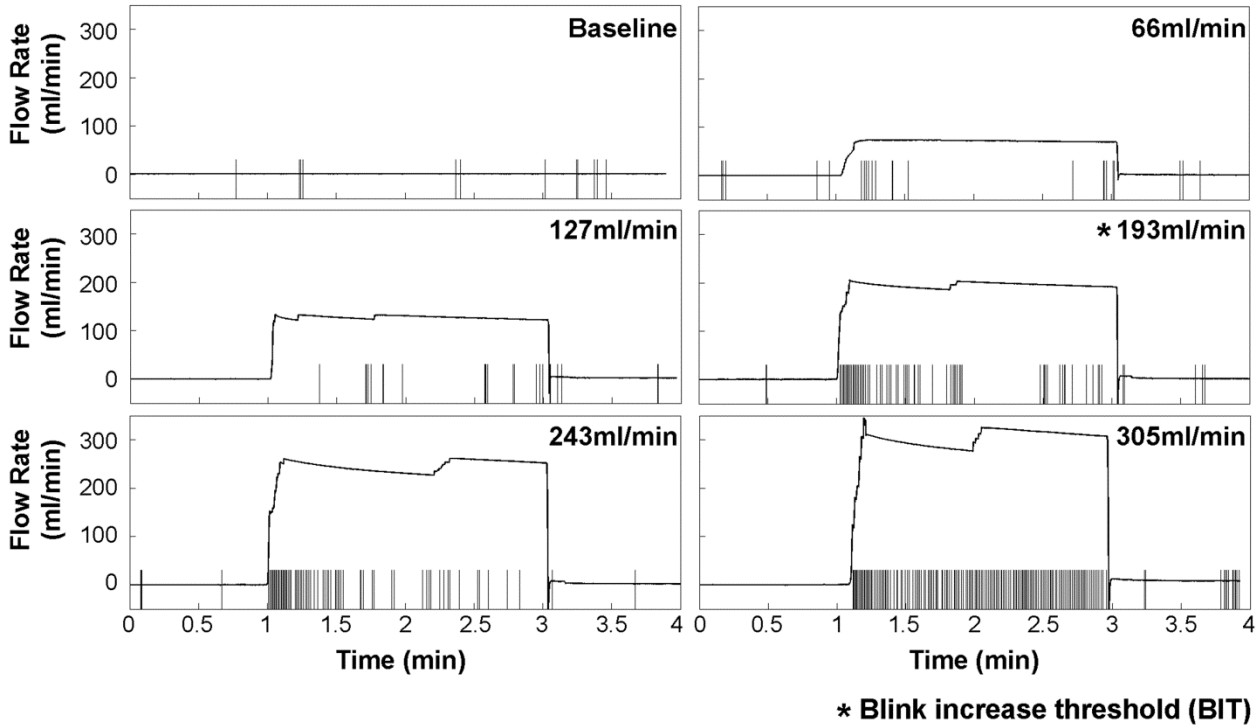

Figure 3: Blink response (Subject 2) to six levels of air stimulation from 0 (Baseline) to 305ml/min. Within each individual graph, the small vertical bars denote the timing of blinks and the horizontal line indicates the flow rate over the trial, with the average flow rate during the central 2 minutes shown at the top right corner. The blink increase threshold (BIT) for this trial is 193ml/min.

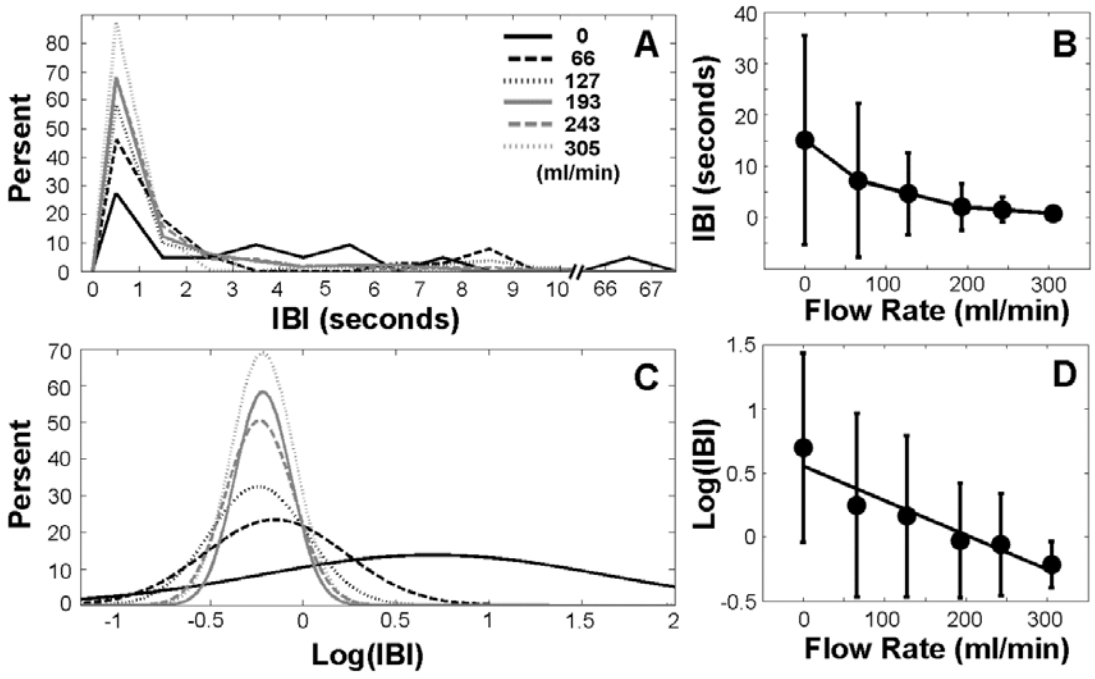

Figure 4: Individual example (Subject 2) using pooled data from three sets of trials. A: IBI distributions under different flow rates. The y axis is the IBI frequency in percent of the total number of IBI data points. B: Mean and standard deviation of IBI as a function of flow rate. C: Log transformation of IBI distributions under different flow rates. Each histogram was fitted with normal curve, and only the best fitted normal curves are shown here. D: Mean and standard deviation of IBI after log transformation as a function of flow rate. A linear regression line was fitted to the data.

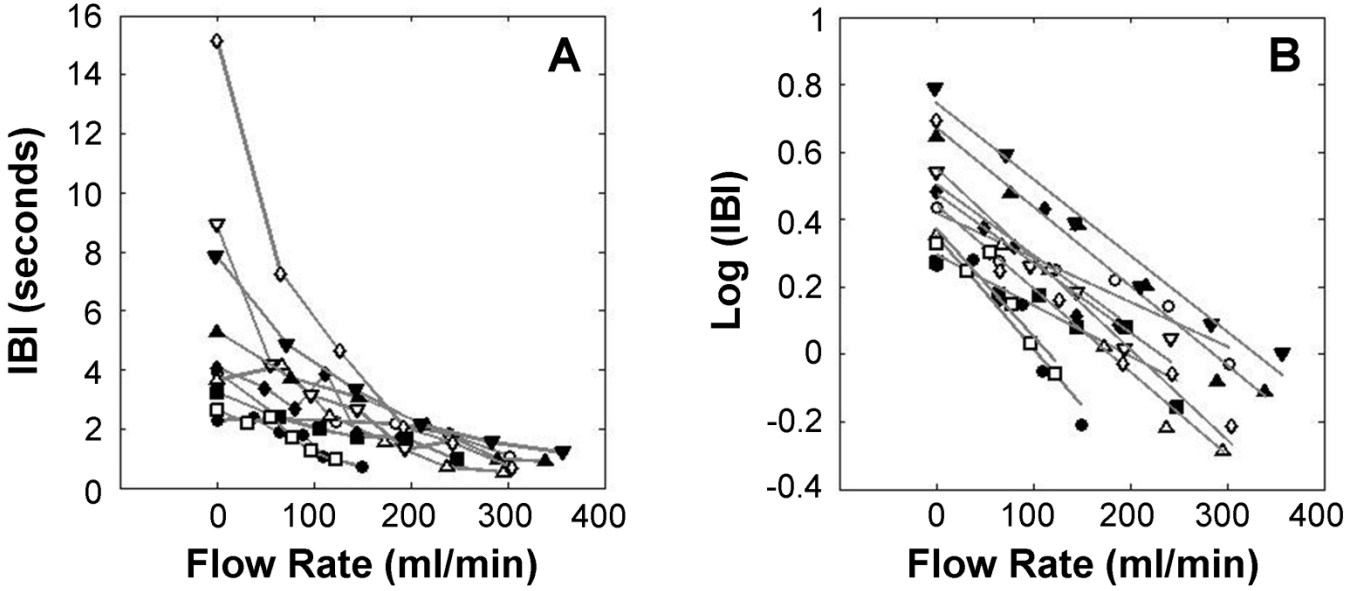

Figure 5: Blink response for all subjects. A: IBI under different flow rates. B: Log of IBI under different flow rates with linear regression line fitted for each subject.

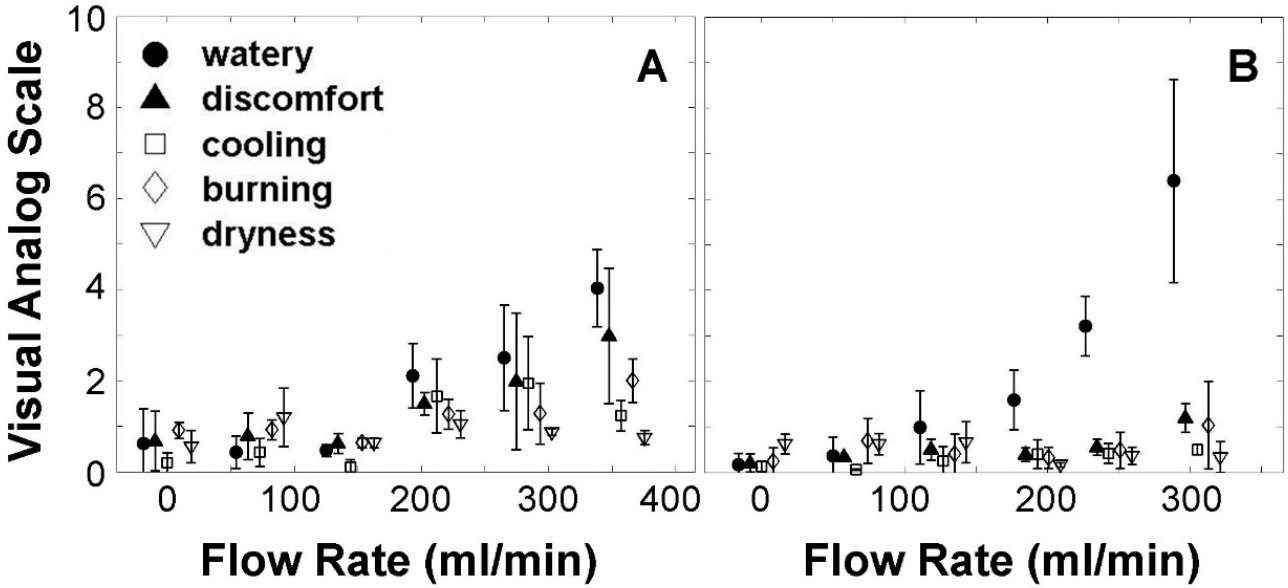

Figure 6: Sensory responses to different flow rates. Markers represent the average sensations from 3 trials with error bars. A: Subject 1. B: Subject 2.

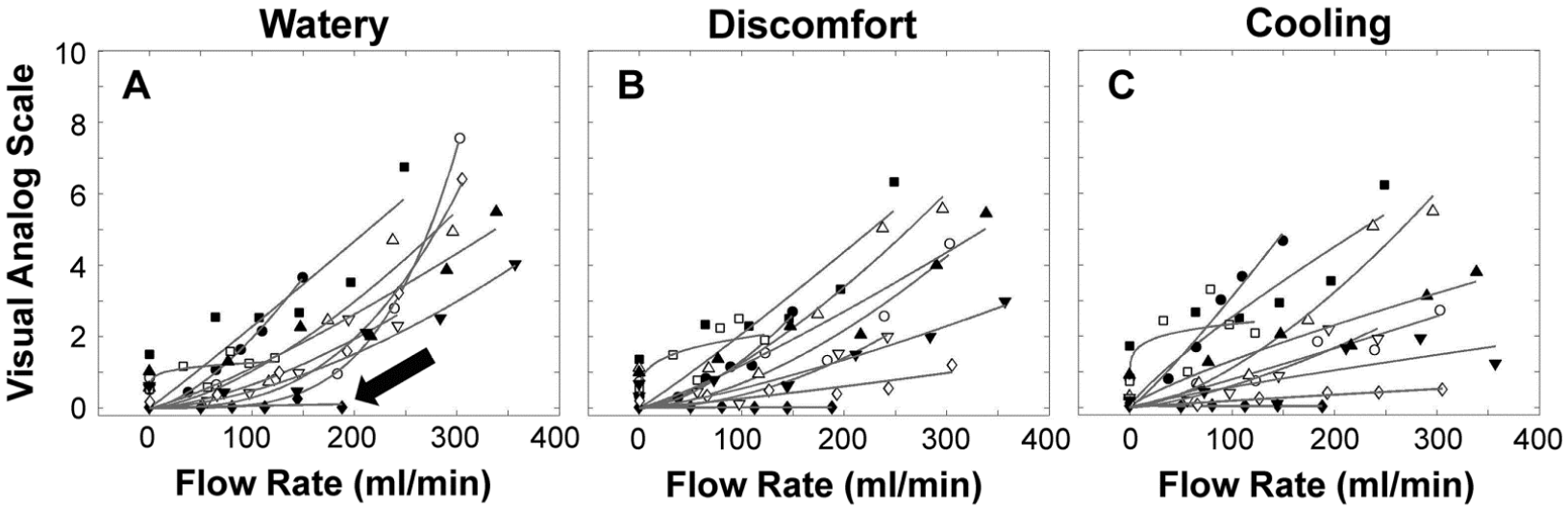

Figure 7: Sensory response of all subjects (fitted with the Stevens' Power function) to different flow rates. Different symbols represent different subjects. A: Watery. B: Discomfort. C: Cooling. The arrow indicates subject with low responses to the stimuli.

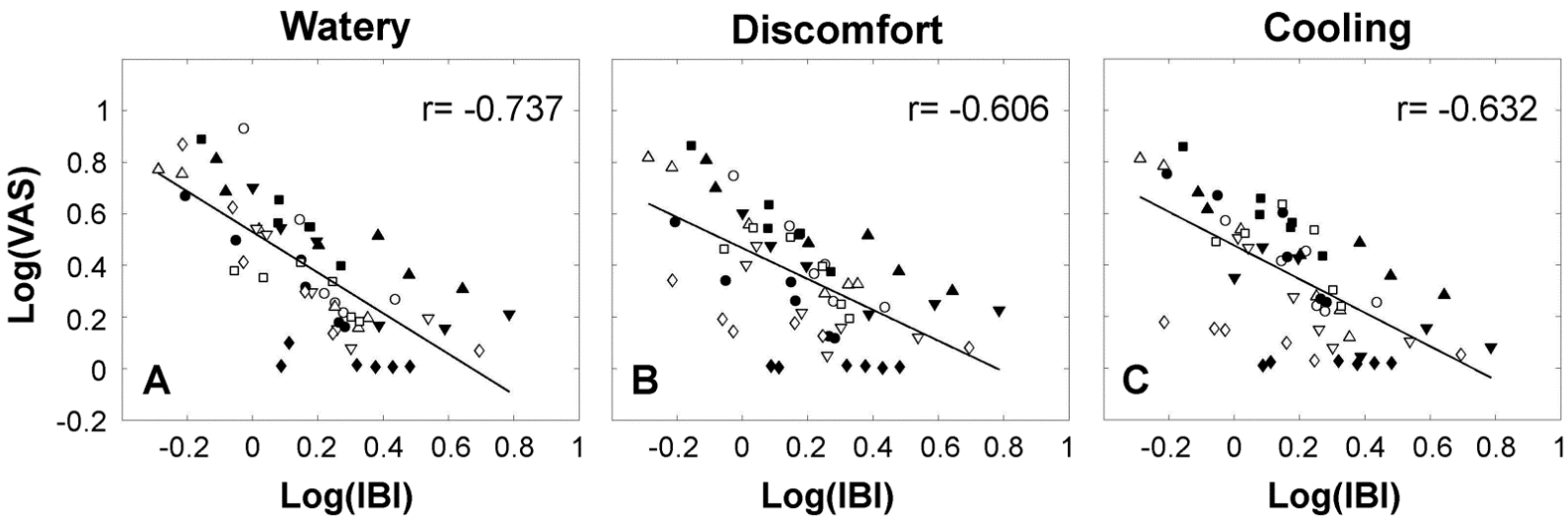

Figure 8: Correlation between IBI and the sensory response. A linear regression line was fitted for each sensation (Pearson's correlation coefficient, $p<0.001$). Different symbols represent different subjects. VAS: Visual analog scale. A: Watery. B: Discomfort. C: Cooling.

CHAPTER IV

THE EFFECTS OF INCREASING OCULAR SURFACE STIMULATION ON BLINKING AND TEAR SECRETION

Abstract

Purpose: The purpose of this study was to determine how increasing ocular surface stimulation affected blinking and tear secretion, while controlling task concentration.

Methods: Ten healthy subjects concentrated on a visual task (computer game), while a custom pneumatic device generated air flow toward the central cornea. Six flow rates (FR) were randomly presented and one microliter 2% fluorescein was instilled before each trial to visualize the lower meniscus. Blink, tear meniscus and air flow were recorded simultaneously. Interblink interval (IBI) and tear meniscus height (TMH) were measured, and tear meniscus fluorescein concentration (TMFC) was calculated from each trial. In order to normalize the individual difference at the beginning of the trail, the relative changes, maximum difference and slope of the TMH and TMFC, were further calculated from each trial. Tear secretion response was separated from the effects of blinking, by calculating the slopes of TMFC change within each IBI (IBI-TMFC slope).

Results: Both blinking and tear secretion were increased with air stimulation, but with several seconds time lag. The mean (±SD) IBI was decreased from 9.47±8.79 during the

baseline to 1.39±1.11sec during the maximum air stimulation. After log transformation, there was a significant linear correlation between surface stimulation and IBI (Pearson's r= -0.471, p<0.001, pooled data). TMH relative changes were poorly correlated with surface stimulation (Pearson's r= 0.396, p<0.002, pooled data), whereas the TMFC relative changes had a much better correlation (Pearson's r= 0.710, p<0.001, pool data). The absolute value of IBI-TMFC slope, which represented the tear secretion rate, was increased from 0.014±0.013 during the baseline to 0.097±0.045 log(%)/sec during the maximum air stimulation. After log transformation, there was a significant linear correlation between surface stimulation and IBI-TMFC slope (Pearson's r= 0.611, p<0.001, pooled data). On average, the new tear shown at the lower meniscus lagged behind the high blink response by 6.54±4.07sec to the stimulus.

Conclusions: Blinking and tears secretion increased linearly with surface stimulation, presumably for the protection of the ocular surface. The newly secreted tear showed up at the lower meniscus a few seconds late after the air stimulation, presumably due to the tear transportation from lacrimal gland to meniscus. The traditional matrix, TMH, might be problematic for quantifying the tear secretion over time, since it is strongly affected by blinking activities. A novel metric, IBI-TMFC slope, was developed to better quantify the tear secretion rate over time separating from blinking.

Introduction

Dry eye is a common disease that affects millions in the United States (Schaumberg, Sullivan et al. 2003; Schaumberg, Dana et al. 2009) and worldwide.(McCarty, Bansal et al. 1998; Uchino, Nishiwaki et al. 2011) It is considered a multifactorial condition driven by tear film instability and hyperosmolarity with possible damage to the ocular surface.(2007) In the normal condition, the ocular surface is mainly protected by two reflex responses, blinking and tearing.(Levin and Kaufman 2011) During a blink, the upper lid wipes the ocular surface, spreading the newly secreted tears over the ocular surface.(Levin and Kaufman 2011) Despite the importance of blinking and tearing in maintaining a healthy ocular surface, the dynamics of these two responses, secondary to ocular surface stimulation, and their interaction with each other, remain unclear.

Many earlier studies had shown that the ocular surface affected blinking. (Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Himebaugh, Begley et al. 2009; Jansen, Begley et al. 2010; Wu, Begley et al. 2013) Surface stimulation, including wearing a contact lens(York, Ong et al. 1971; Jansen, Begley et al. 2010) or having dry eye,(Tsubota, Hata et al. 1996; Himebaugh, Begley et al. 2009; Ousler, Abelson et al. 2014) significantly increased the blink rate, probably due to the ocular surface irritation involved in both. Conversely, using lubricating eye drops(Acosta, Gallar et al. 1999) or anesthetics,(Nakamori, Odawara et al. 1997; Naase, Doughty et al. 2005; Borges, Garcia et al. 2010) which would be expected to reduce ocular surface neural stimulation, significantly decreased the blink rate. Furthermore, we have previously shown a dose

response relationship between blinking and pneumatic ocular surface stimulation. These results suggested that ocular surface neurons were able to both detect and quantify a surface stimulus on the surface, and then modulate the blink response appropriately (Wu, Begley et al. 2014).

Along with blinking, the ocular surface plays an important role in regulating the tear secretion.(Stern, Gao et al. 2004; Situ and Simpson 2010) This is often measured clinically by the Schirmer's tear test. However, this method provides stimulation in itself and shows poor repeatability.(Lee and Hyun 1988; Nichols, Mitchell et al. 2004) Tear meniscus height (TMH) and fluorophotometry have also been used to study tear secretion in dry eye and other conditions,(Situ and Simpson 2010; Tung, Perin et al. 2014) but few have investigated the dynamic tearing response secondary to ocular surface stimulation.

Stimulation of the cornea is detected by corneal neurons that project to the trigeminal complex in the brainstem through trigeminal afferent fibers. Here, pathways for the blink response and tear secretion diverge. Blink pathways project to the facial motor nucleus to trigger blinks, while tearing pathways project to the superior salivatory nucleus and finally to the lacrimal gland.(Stapleton, Marfurt et al. 2013) Thus, both the reflexive blinking and tearing response can begin with corneal stimulation, although a complete understanding of neural types involved remains unclear.

It is likely that both reflex blinking and tearing in response to corneal stimulation are mediated by the activities of different types of receptors. The air flow can induce a mechanical pressure, as well as hyperosmolarity and cooling due to the evaporation, thus activating the mechanical, thermal and polymodal receptors in the ocular surface. Recent

studies have shown the cold thermoreceptor could respond to the temperature and hyperosmolarity change, and thus, may be involved in producing both reflex and so-call basic tears.(Parra, Madrid et al. 2010; Belmonte and Gallar 2011; Parra, Gonzalez-Gonzalez et al. 2014) In addition, mechanical and polymodal nociceptors might be also involved in triggering reflex tearing. (Belmonte, Aracil et al. 2004) Although the neuron activities under different types and amount of stimulation have been studied in the past decades,(Belmonte and Giraldez 1981; Gallar, Pozo et al. 1993; Hirata and Meng 2010; Hirata and Oshinsky 2012; Parra, Gonzalez-Gonzalez et al. 2014) few studies monitored the final output, tear secretion, under different conditions.

Since both blinking and tearing are triggered to protect the surface, they should interact with each other in a manner that maximizes their protective functions.(Gaffney, Tiffany et al. 2010) However, we are not aware of human or animal studies investigating both responses simultaneously. We hypothesize that tear secretion will increase with surface stimulation, as does the blink response,(Wu, Begley et al. 2014) and that the two responses will be correlated due to their common origin at the ocular surface.(Stapleton, Marfurt et al. 2013) Therefore, in this study, we developed a human-based laboratory model to investigate the effect of varying levels of ocular surface stimulation on blinking and tear dynamic at the lower meniscus over time. Both the timing and amplitude of these responses were measured simultaneously to understand how they interacted with each other. Only young and healthy subjects were recruited to determine the response within a normal physiological range to avoid any potential effects from corneal neurons altered in disease.(Bourcier, Acosta et al. 2005; Situ, Simpson et al. 2008; Tuisku, Konttinen et al. 2008; Basuthkar Sundar Rao and Simpson 2014)

Methods

1. Subject

The study was conducted at the Borish Center for Ophthalmic Research at the Indiana University School of Optometry, Bloomington, Indiana. It adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at Indiana University. Informed consent was obtained from each subject prior to beginning the study.

Ten young, healthy subjects were recruited. Subjects reporting ophthalmic disorders, including dry eye, ocular allergies, contact lens wear or any systemic disease were excluded. At the beginning of the study, subjects were told that the reason for the study was to examine the tear film on the front of the eye while they were engaged a computer task. They were *not* informed that the purpose also included monitoring of blink or tear secretion until the study was completed to avoid any potential cognitive contaminating effects on blinking or possible tear secretion.(Doane 1980)

2. Experimental Procedures

There were two visits in this study. The purpose of the first visit was to estimate the level of pneumatic stimulus that triggered an increased BR to determine the air stimulus levels for each individual, and then experimental data were collected during the second visit. During the first visit, subjects filled out the Dry Eye Questionnaire

(DEQ)(Begley, Caffery et al. 2002) to assess habitual symptoms of ocular irritation and dry eye. Then, they were seated behind a slit lamp biomicroscope (Zeiss 20SL, Carl Zeiss, Germany, 12x magnification) with two cameras for simultaneous recording subject's lower meniscus (GRAS-20S4C-C, Point Grey, Germany, 7.5Hz) and the upper lid movement during blinking (Basler piA640-210gm, Basler AG, Germany, 30Hz).

In order to record blinking, an adhesive 2 mm diameter reflective white dot (3M Company, Minnesota, USA) was gently positioned as close as possible to the margin of the upper lid. One microliter of 2% fluorescein was instilled into the lower meniscus in order to monitor its height and fluorescence over time. Subjects played a computer game viewed through a beam splitter. The tested eye was positioned so that subjects looked down slightly (approximately 5°) and the inferior meniscus was viewed over the cornea (not limbus or conjunctiva) where visualization of the meniscus was best for image analysis purposes. The lower lid and tear meniscus were illuminated using the slit lamp biomicroscope light source with a cobalt blue filter positioned at an angle of 30° temporal and a Wratten number 8 filter over the observation port. The illuminated area was below the pupil to avoid initiation of reflex tearing by the bright light. Only the right eye was tested. The other eye was manually held shut by the subject.

An instrument similar to a pneumatic esthesiometer was used to stimulate the cornea with air flow.(Belmonte, Acosta et al. 1999; Situ, Simpson et al. 2008) The air stimulus was aimed at a slight angle from the center of the cornea (12° from horizontal and 5° from vertical), so that it did not block the slit lamp view of the cornea or the subject's vision while playing the computer game. The distance between the tip of the air stimulus and the cornea was 15mm. Its position was constantly monitored by a calibrated

side mounted camera. The air intensity from the stimulus tip was recorded by a customized LabVIEW 5.1 program and time stamps were used to match the air stimulus with BR and tear meniscus data.

It was necessary at the outset to determine the levels of pneumatic air stimulation to be used in the experiment for each subject because subjects varied in their sensitivity to the stimulus. To obtain baseline measures, subjects were seated behind the slit lamp biomicroscope and instructed to play the computer game for two minutes without any air stimulus (Figure 1a). The air stimulus was then systematically increased in a step size of 50ml/min every 30 seconds until the observer noted an increase in BR, when the trial was stopped (Figure 1b).

A customized MATLAB® (The Mathworks™, Natick, MA) program was used to track the white dot on the upper eyelid and determine whether a blink occurred.(Freudenthaler, Neuf et al. 2003) If the marker reached or went over the middle level of the eye aperture, it was counted as a blink. All lid movements that extended to this point in the eye aperture were treated as blinks, but lesser partial blinks that did not extend to this point were not counted as blinks. The extent of each blink was not recorded, only its occurrence.

The two minutes baseline trial was segmented into four half minute windows and the BR in each window was calculated (Figure 1a), averaged and a standard deviation between measures obtained (Figure 1c, error bar). The air stimulus that significantly increased the BR was defined as three standard deviations higher than the mean value from the four windows during the baseline (Figure 1c, circled). This air stimulus level

was used to set the six levels of pneumatic stimuli to be tested next visit by multiplying it by 0, 0.25, 0.5, 0.75, 1.00, and 1.25; thereby estimating a range of sub- to supra-threshold stimuli producing an increased BR for each subject.

During the second, experimental visit, the effect of the six levels of pneumatic stimuli on the BR and tear secretion was determined. As in the first visit, a small white dot was placed on the upper eyelid to monitor upper lid movement to measure the BR. A small black dot was drawn in the center of the lower eyelid using a cosmetic eye liner (Maybelline® Eyestudio master precise, black) to enable registration of the lower meniscus images (see tear meniscus analysis). One microliter of 2% fluorescein was instilled into the right eye before each trial. Each stimulus level trial began with 30 seconds of no stimulus, followed by one minute of the stimulus, followed by 30 seconds without stimulation. Stimulus levels were presented in a random order, including the baseline measurement, which involved monitoring subjects for two minutes without stimulation. There were five minute breaks between trials.

3. Blink Analysis

During each trial, the blink activity and air flow were overlaid by using the time stamps. Both IBI and BR were calculated under different air stimulation.

In order to measure the time point that blink frequency was significantly increased after air stimulation, a spike density analysis was used to transform the raw blink data.(Szucs 1998) This method was chosen because it is sensitive to rapid, local changes

in an event such as neuron firing or, in our study, a blink. Figure 2 shows an example of neural responses within one second where each vertical bar represents a spike.(Wallisch, Lusignan et al.) In this analysis, each response (event) bar is replaced with a normal curve (Figure 2, blue arrows). Curves are additive, so that closely adjacent curves create a higher peak (Figure 2, red arrow). The spike density analysis provides a method quantification of the time that blinking started to increase in response to the stimulus. It allowed us to quantify the blink increase time without losing the temporal resolution.

Figure 3 shows how the spike density analysis was applied to blink data in this experiment. We chose an interval of -0.3 to 0.3sec to mark events (blinks). Therefore, each blink was replaced with a normal curve, centering at the blink (event) time point and spreading over a range of time value from -0.3 to 0.3sec and thus was transformed into a continuous curve. Figure 3 represents an example with the vertical dashed lines (blue) representing the time when the air was on and off. Both the mean and standard deviation of the blink curve before the stimulus were calculated. Due to the high variation of the blink activities, the blink increased time was defined as a blink curve higher than three standard deviations (horizontal blue line) from the mean baseline value (Figure 3, arrows).

4. Tear Meniscus Height Analysis

In this experiment, we hypothesized that tear secretion would increase with air stimulation; thus, the TMH should also increase with increasing air stimulation. The following image analysis was used to measure the TMH.

Due to the constant movement of meniscus, all the frames were first registered by the black marker on the lower lid to align the meniscus in the same location across trials (After Effects, Adobe, USA). A customized Matlab® program was used to create a rectangular region of interest (ROI) centered on the lower meniscus in the first image of the trial (Figure 4a, white box). The rectangle was set with fixed width but adjustable height, so that the meniscus was always within this region across trials. If there were light reflections along the lower meniscus (Figure 4a, white arrow), rectangle was moved in a nasal direction (up to 3mm) until no light reflections fell inside the ROI.

Each column within the resulting ROI matrix contained meniscus height information, but these could not be average directly to obtain the TMH because the meniscus was usually slanted at an angle within the ROI (Figure 4a). In order to use TMH information from all columns within the ROI, a cross-correlation between columns was performed to align the meniscus and remove its tilt. To do this, the pixel intensity value was plotted as a function of the vertical position in each column within the ROI (Figure 4b). The intensity curve from each column was then cross-correlated with that in the first column and the shift required for alignment was computed. Each ROI column was moved vertically to overlap with the first column maximally (Figure 4c). The average pixel intensity at each vertical location was then calculated (Figure 4c, red line). By using this method, we were able to summarize the information from a two dimensional image (Figure 4a) into a one dimensional matrix (Figure 4c). In addition, it significantly decreased image noise, such as fluorescein spots spilled on the lower lid (Figure 4a, red arrow; Figure 4c, circle).

Next, blinks and large eye movements were discarded from the data. To do so, a second cross-correlation was performed to compare each subsequent image with the first image in the trial. If the image contained the meniscus, the cross-correlation with the first image would be high, otherwise it would be very low. The difference between each cross-correlation value and the previous one was then calculated. Due to the variability of the image before and after blinks, thresholds of 1/20 of its minimal and 1/10 of its maximum cross-correlation values were set to identify and remove blinks and eye movements. In order to check whether all the blinks images were identified and discarded, a color map was generated (Figure 5). The x axis is frame number, and the y axis was vertical position within ROI. The pixel intensity is represented by color. Figures 5 showed an example before (Figure 5a) and after taking blink frames out (Figure 5b).

TMH was measured using a threshold method on the first 30 seconds of images (pre-stimulus) from each trial. Theoretically, the upper edge location of the tear meniscus should be similar during the first 30 seconds, which no stimulus was applied (Figure 6a-b, left side of the dashed line). The upper edge threshold was determined when the standard deviation of the upper edge locations was less than 2 during the pre-stimulus period (Figure 6a, black line). For comparison, an example of a lower threshold is shown by the white line in Figure 6b. In this case, there was more fluctuation of the upper edge location due to the noise on the cornea. A similar analysis was used to find the lower edge of the meniscus, except that the criterion was 4, due to the high noise/signal ratio in the bottom region of the ROI. After finding the upper and lower edges of the meniscus, the TMH was calculated and converted into millimeters.

5. Tear Meniscus Fluorescein Intensity Analysis

The fluorescein intensity changes during trials noted during the TMH analysis led us to develop a new tear secretion metric based on fluorescein intensity in the lower tear meniscus. It is based on the idea that, as new tears are secreted, they enter the meniscus and dilute the current fluorescein concentration, thus changing its intensity over time. However, tear meniscus analysis of fluorescence intensity is potentially complicated by the phenomenon known as concentration quenching. Quenching is widely used in biology to study the cell membrane permeability and volume changes within cells.(Solenov, Watanabe et al. 2004; Ruiz-Ederra and Verkman 2009) Figure 7a illustrates this effect. As the concentration of fluorescein rises, fluorescence intensity increases up to a maximum, but then the intensity decreases sharply when the concentration is higher than 1 mg/ml (0.1%). This phenomenon, which is called quenching, is due to the light absorption between closely adjacent molecules, leading to low levels of fluorescence when the concentration of fluorescein is high. Thus, measured fluorescein intensities could theoretically represent concentrations either lower or higher than maximum, rendering two solutions possible and complicating an analysis of fluorescein intensity measures over time. In addition, some trials began with apparent quenching, further confounding the results of a direct intensity analysis.

For this reason, we calculated tear meniscus fluorescein concentration (TMFC) from intensity using a previously established mathematical model relating fluorescein concentration and intensity: (Nichols, King-Smith et al. 2012)

$$I = k^* (1/(1+ (f/f0)^2))^* (1- e^{(-a^* h^*f/weight^*10)}); \quad \text{Equation 1}$$

where I is the fluorecein intensity, k is a constant equal to 1; f is fluorescein concentration (g/100ml); f0 is the critical fluorescein concentration (0.2 (g/100ml), a is a molar extinction coefficient (cm^(-1)M^(-1)), h is the tear film thickness (cm) and weight is molecular weight of fluorescein (376 g/M). Calculations using this model created a very similar function (Figure 7b) when compared to the classical fluorophotometry results of Webber and Jones (Figure 7a).(Webber and Jones 1986)

In this study, the meniscus depth or thickness (h) was not measured directly, but was estimated from the TMH. Figure 8a (triangle) is an example of meniscus depth, showing a cross-section of the tear meniscus using an OCT.(Srinivasan, Chan et al. 2007) According to previous studies, the TMH and depth are quite variable among individuals, but the ratio between height and depth is relatively constant, with an average ratio of 1.56± 0.16. (Zhou, Li et al. 2009; Ibrahim, Dogru et al. 2010; Bujak, Yiu et al. 2011; Qiu, Gong et al. 2011; Tittler, Bujak et al. 2011) Thus, we estimated meniscus depth (h) from the TMH by dividing by 1.56.

The fluorescein intensity value used in Equation 1 also needed to be determined. Figure 8 showed an individual example of fluorescein image (Figure 8b) and its intensity curve (Figure 8c) along the vertical position within ROI, demonstrating that fluorescein intensity within meniscus was not even, theoretically due to variations in tear film thickness within the meniscus. Because the maximum fluorescein intensity would be expected at greatest meniscus depth, the maximum value of fluorescence intensity across the meniscus was used for each time point in Equation 1 calculations. The fluorescein intensity in pixel value was then standardized by its maximum intensity within each individual subject before applying into the equation.

When the TMFC was calculated for each time point, both low and high concentration values were generated. Because the TMFC should only decrease over time, if the intensity changed from low to high, then that should be due to the quenching effect and the starting point of the TMFC should be at the high concentration end. Otherwise, the TMFC should begin at the low concentration end of the intensity curve. If the intensity didn't change during the whole trial, the TMFC was randomly assigned to the high or low concentration end. (Figure 9C)

6. Validation of the TMFC Calculation in an Eye Model

In order to verify the TMFC calculation and to determine the relationship between fluorescein intensity and concentration in our slit lamp biomicroscope setup, we constructed an eye model to mimic the lower tear meniscus (Figure 9). In the lower meniscus eye model (Figure 9a), the curvatures of the upper and lower circles were 7.8 and 12mm, creating an angle of 76.46 degrees. (Srinivasan, Chan et al. 2007) Two microns of 48 different fluorescein concentrations (from 0.001 to 1%) were pipetted into the eye model meniscus and imaged under the same experimental conditions as the human subjects in this study. Figure 9b shows some of these images. Intensity data were fitted using Equation 1 and a best fit curve was generated (Figure 9c, blue line, R-square= 0.9807).

7. Tear Meniscus Height and Concentration Analysis

In each trial, two graphs were generated to show all the information recorded (Figure 10). The dashed lines represent the timing for the air stimulus, which separated the trial into three periods: pre-stimulus, during-stimulus and post-stimulus periods (Figure 10b). The corresponding color map was also shown for easy visualization of the tear meniscus.

Figure 11 shows the parameters measured from the tear meniscus. For TMH, the relative change was calculated (max difference) to normalize the individual difference during the per-stimulus period. The TMH increase time was defined when the TMFC was increased 1/10 of its maximum difference. The TMH slope was calculated as the slope from the TMH increase time point to the maximum slope value. If there was no obvious change in the tear meniscus, the TMH slope would be calculated in the whole during-stimulus period. The TMH increase time would not be calculated when the TMH slope was too small (less than 0.01 mm/sec). The same analysis was done for the log TMFC.

8. Changing Rate of Fluorescein Concentration within Interblink Interval

In order to study the tear secretion response more directly, the TMFC slope within each IBI (IBI-TMFC slope) was also calculated (Figure 12). Theoretically, if there was no or minimal tear drainage between blinks, the TMFC would decrease due to newly secreted non-reflex tears entering the meniscus. Thus, the slope of the TMFC between blinks would be expected to be minimal. However, if reflex tearing occurred, tear secretion should act to decrease the TMFC faster between blinks, creating a greater slope of change.

To perform this analysis, the TMFC data were first segmented by the blink time (Figure 12a). A linear slope was fitted to the TMFC from each IBI segment and the slope was calculated (Figure12b). The final output was the slope of the TMFC within each IBI, which allows isolation of tear secretion from IBI. If there was no or minimal tear secretion, the TMFC slope would be close to zero. If there was big tear secretion, the TMFC slope would be more negative. The resulting value was plotted with the IBI (Figure 12c). Images with tear film fluorescence that was too low to analyze (i.e. <0.0067% (-5 log(%)) were excluded.

9. Statistical Analysis

Both TMH and TMFC were smoothed using the loess method with a span of 5% to reduce noise. The Pearson's correlation coefficient was used to determine the association between the pneumatic stimulus and IBI. The exponential function was used to fit in between pneumatic stimulus and IBI-TMFC slope.

Results

1. Subject

The average (±SD) age of study subjects was 23.9±1.7 years (range: 22 - 27 years). Five were female and five were male. The median DEQ-5 score(Chalmers, Begley et al. 2010) was 3 (range: 0-7) and none of the subjects reported a previous dry eye diagnosis on the DEQ or thought they had dry eye.(Begley, Chalmers et al. 2003) The average (±SD) Schirmer's I tear test and TBUT were 16.1±13.2 mm/5min (range: 1-

35mm) and 7.2±5.9 seconds (range: 2-22 seconds), respectively. The average (± SD) of the temperature and humidity in exam room were 24.2±1.1° C and 44.0±1.6%, respectively.

2. Individual Example

Figure 13-15 show the data from Subject 6. In Figure 13, the trials are presented from baseline to maximum stimulation with the air intensity labeled (de-randomized for presentation). The color map above each graph provides a visualization of the changes in meniscus intensity and height over time. The black vertical lines at the bottom of each graph indicate the timing of the blinks and the dotted lines show the pre-stimulus, during-stimulus and post-stimulus time periods. The red line in each graph is the TMH and the blue line is the TMFC. The subject in Figure 13 showed a high BR even during baseline, so that the average IBI (± SD) was 1.87±1.13 and the BR was 31 blinks/min. The IBI, the TMH and the TMFC appeared to change slightly at 87ml/min and markedly by 109m/min. The IBI-TMFC slope also followed this trend (Figure 14).

The graphs in Figure 15 summarize all of these parameters for Subject 6. The IBI decreases with increasing air stimulation, as we found in our previous studies. (Wu, Begley et al. 2014; Wu, Begley et al. 2014) The TMH and TMFC maximum difference increased or stayed the same with increasing air stimulation, while the TMH and TMFC slope decreased at the last level of air stimulation. The results for the IBI-TMFC slope was much clearer compared to TMH and TMFC, probably because that measure rules out

the blink effect. The exponential function was fitted nicely between air intensity and the average IBI-TMFC slope (r square= 0.975, Figure 15e).

The time points for increased blinking, TMH and TMFC were also interesting. These time points were graphed for the last three trials for Subject 6 in Figure 15f. In this subject, the BR increased very quickly after the stimulus, with an average blink increase time of 0.95±0.89sec. However, the TMH and TMFC changed a few seconds later. The average TMH increase time and TMFC decrease time were 5.61±1.29 and 4.56±0.10sec, respectively.

Figures 16-18 show the responses of another subject (subject #9) whose BR was much lower initially, compared to the previous subject. The IBI during the baseline was 10.92±7.83 sec and BR was 5 blinks/min. With increasing air, the IBI was decreased somewhat at 237ml/min and dramatically by 290ml/min (Figure 16a-f and Figure 18a). The TMFC showed similar changes, especially at 290ml/min where fluorescein dye washed out quickly, causing it to be impossible to measure TMH later in the trial (Figure 16f). For the TMH, both max difference and slope were slightly increased with air stimulation, and the change between trials was much smaller compared to previous subject (Figure 18b). However, as before, there was a good exponential fit between air intensity and IBI-TMFC slope (r square= 0.905, Figure 17 and 18e).

Similar to the previous subject, there was a difference between the time to blink increases and the tear metrics. When the BR increased, it occurred almost immediately after the air stimulus turned on with a blink increase time of 0.001 and 0.002sec during the last two trials. Tear meniscus times were later, with TMH at 2.77 and 0.86sec, and TMFC times at 6.76 and 10.84sec during the last two trials (Figure 18f).

3. Blink Response to Air Stimulation

Figure 19a shows the aggregate data from all subjects. The blink activity was highly variable between subjects during baseline measures that the average IBI was 9.47±8.79sec. The IBI tended to decrease and BR increased with air stimulation, except two subjects whose IBI did not decrease in the first three trials, but still decrease a lot with higher air stimulation. That was probably due to the highly irregular blink responses on these two subjects during the baseline and relative small sample size (one minute) in this study. Under maximum air stimulation, there was much less inter-subject variation in the IBI (average IBI was 1.39±1.11sec). Figure 19b shows the log IBI versus log air intensity for all subjects. Eight out ten subjects had significant linear correlation between air intensity and the log IBI ($p<0.05$), with an average slope was -0.0096± 0.0049, ranging from -0.0033 to -0.0177 (eight subjects).

4. Tear Secretion Response to Air Stimulation

4.1. Tear Meniscus Height (TMH)

Figure 20a shows the average TMH for all the subjects during all the trials. As we expected, the TMH was variable between subjects that the average TMH at the beginning of the trials was 0.25±0.07mm. This individual variation was normalized by calculated the relative change within trial (TMH max difference: the maximum minus the minimum values of TMH within each trial) and the aggregate data was shown in Figure 19b. TMH max difference was highly variable between subjects that the average TMH max

difference was 0.41± 0.39 mm, ranging from 0.07 to 1.12mm. Five out of 10 subjects showed increased TMH max differences with increasing air stimulation, but the other half of the subjects showed only minimal changes in the TMH. Figures 13 and 16 show examples of each type of subject. Three subjects showed a large TMH max difference at one level of tear secretion, but this decreased with the next level(s) of air stimulation (Figure 20b). This led to a poor overall correlation between air stimulation and TMH max difference ($r= 0.3963$, $p=0.0017$, pooled data).

Similarly, the TMH slope showed high variability among subjects and did not always increase with increasing air stimulation (Figure 20c). The average TMH slope from all the subjects was 0.073±0.076 mm/sec and ranged from 0.004 to 0.251 mm/sec. The overall correlation was also low ($r= 0.3921$, $p= 0.0019$, pooled data).

4.2. Tear Meniscus Fluorescein Concentration

Due to the difference in tear volume and the order of the trials, the absolute TMFC values were quite variable between subjects (Figure 20d). The maximum difference, which was the relative change within a trial, was calculated to normalize this variation, and the result was much clearer. The relative change of TMFC showed an increase with surface stimulation for all subjects (Figure 20e). Individual variation between subjects was also relatively small. The TMFC maximum difference for all subjects was 2.86± 0.98 log(%), ranging from 1.03 to 3.94 log(%). Six out of ten subjects had significant linear relationship between air intensity and TMFC max difference that r ranged from 0.853 to 0.963 ($p<0.05$, 6 subjects). There was a significant

linear relationship between stimulus air intensity and TMFC maximum difference when pooling all the data together ($r= 0.710$, $p<0.001$, pooled data).

The TMFC slope showed high individual variation among subjects and a variable response to air stimulation within subjects (Figure 20f). The average TMFC slope was 0.500±0.659 log(%)/sec, ranging from 0.058 to 2.267 log(%)/sec. The correlation between air intensity and TMFC slope was also highly variable between subjects that only four subjects had a significant correlation (Pearman's $p<0.05$, r ranged from 0.824 to 0.946).

Because the TMH and TMFC are both affected by blinking, the TMFC results were much clearer when ruling out the blink effect by examining the slope within each IBI. An exponential function was fitted between air intensity and the average IBI-TMFC slope within each subject (Figure 21). Nine out ten subjects showed good fitting with exponential functions (r square >0.65), and one subject couldn't fit in the exponential function (r square= 0.045). The individual variation was high that the average exponent value was 0.014±0.011, ranging from 0.003 to 0.032 for the nine subjects.

4.3. Response Time Points for Blinking and Tear Secretion

Figure 22 (a-c) shows the time points when all subjects showed increased blinking, TMH, and a decreased TMFC in response to air stimulation. The increase in blinking was very quickly after the air stimulus, with an average blink increase time of 0.60±1.12sec. The tear secretion measures occurred later, with an average TMH increase time of 6.30±4.80sec and a TMFC decrease time of 7.29±3.95sec. The average time difference between blink and tear was 5.91±5.37 sec for all the subjects all the trials.

These data are plotted together in Figure 22, illustrating that blinking was the earliest response to air stimulation.

4.4. Comparing Blinking and Tear Secretion

The tear dynamic at the lower meniscus mainly depends on the input (tear secretion) and output (tear drainage). As mentioned before, we were assuming the IBI-TMFC slope represented the tear secretion rate, since it ruled out the blink effects and its change should be mainly due to the newly secreted tear. BR, rather than IBI, was analyzed here to represent the blink response since the BR is positively correlated with tear drainage.

In order compare the blink response and tear secretion results, data were combined from all subjects. To do so, each trial was segmented into 60 windows (each window was ~2sec) and the average IBI-TMFC slope and BR were calculated within each window, for each stimulus air intensity level. The 2 seconds window was picked since it was long enough for calculating BR, and short enough to catch the transient change for IBI-TMFC slope.

Figure 23 shows these results (red: IBI-TMFC slope, blue: BR). The horizontal dashed lines represent three standard deviations from the mean value during the pre-stimulus period.

Similar to the individual examples in Figures 13-18, the BR and IBI-TMFC slope did not change much during the first three trials, although there may be some small tear secretion occuring during the third trial (Figure 23c). However, with increasing the stimulus intensity, both blinking and tear secretion were significantly increased with

increasing air intensity. The maximum IBI-TMFC slopes were: -0.0346, -0.0718, -0.0968, -0.1585, -0.3391, -0.4777 log(%)/sec from baseline to maximum stimulation and the maximum BR were: 21, 27, 30, 78, 87, 114 blinks/min.

Figure 23 also illustrates the time difference between the blink and tear secretion responses with increasing air stimulation. In Air Levels#4-#6, it is clear that the blink response occurred first. In addition, both responses appear to show adaptation in these aggregate results. Both blinking and tear secretion decrease even during the during-stimulus period.

Since both blink and tear secretion are triggered by inputs from the ocular surface, it is reasonable to hypothesize that the responses could be correlated in some manner. Figure 24 shows the relationship between the IBI and the IBI-TMFC in log space for all subjects and all the trials. Individual variation was very high and a significant correlation was only found on four out of ten subjects (r ranged from -0.812 to -0.957, Figure 24a). With pooled data, the overall correlation was modest ($r= -0.556$, $p<0.0001$, Figure 24b).

Discussion

This study supported the hypothesis that ocular surface stimulation increased both blink rate (Wu, Begley et al. 2014) and tear secretion, (Situ and Simpson 2010) presumably for protection of the ocular surface. It represents the first, to our knowledge, to investigate both the blink and tear secretion response to ocular surface stimulation and their potential interaction with each other. We found that most subjects demonstrated a linear increase in the blink response and an exponential increase in tear secretion with

ocular surface pneumatic stimulation, although the individual variation was high. The greatest increase in blink rate occurred almost immediately after stimulation, whereas new tear secretion appeared in the meniscus a few seconds later for most subjects. Both blinking and tear secretion showed adaptation to the stimulus, in that both returned to near baseline levels while the stimulus was still present. These results imply that, while ocular surface air stimulation was evidently involved in inducing blinking and tear secretion, the differing nature of the responses may suggest that dissimilar ocular surface neurons were stimulated to precipitate the responses. It is also possible that the nature of the motor and automatic response itself (blinking versus tear secretion) was involved in producing the underlying differences in the timing and appearance of each response.

To identify the time course of tear secretion and separate its effects from blinking, we developed a novel metric based on calculated inferior meniscus fluorescein concentration, the TMFC. Traditionally, the TMH has been used to estimate tear volume (Mainstone, Bruce et al. 1996; Kawai, Yamada et al. 2007; Tung, Perin et al. 2014) or secretion,(Koh, Tung et al. 2012) but we found that increased blinking complicated TMH results in some subjects (Figure 20a-b). This is presumably because blinking and tear secretion have opposing effects on the TMH. Tear secretion acts to increase the tear volume as well as the TMH,(Situ and Simpson 2010) whereas blinking promotes drainage of the tears and thus decreases the TMH.(Sahlin, Laurell et al. 1998; Johnson and Murphy 2006) Thus, increased blinking may act to “mask” tear secretion as measured by TMH. This is most likely the reason why five out of the 10 subjects in this study showed no obvious increase in TMH with air stimulation at any level, despite their increase in blinking. In addition, the TMH became very difficult to measure when there

was insufficient fluorescein dye present following excessive tear secretion (Figure 16f). Thus, the use of TMH as a metric of tear secretion resulted in potential inaccuracies due to interactions with blinking and frequent missing data in the second half of many trials.

For this reason, we developed metrics for tear secretion based on calculated changes in fluorescein concentration, rather than TMH. As is obvious with a visual inspection of the effects of pneumatic stimulation in Figures 16e-f, fluorescein dye concentration changed markedly, while TMH did not. Thus, we searched for methods that allowed calculation of fluorescein dye concentration from changes in fluorescence intensity (Nichols, King-Smith et al. 2012; Braun, Gewecke et al. 2014) and employed these methods to develop novel fluorescence metrics for tear secretion from pixel intensity in our images. One potential complication in this analysis is that two fluorescein concentrations can be calculated from one intensity value, one below (low concentration) and one above the peak concentration (high concentration in the quenching regime). We solved this problem by noting the trend of fluorescence intensity changes over time (see Methods section). We believe we were able to assign the corrected fluorescein concentration when there was noticeable change in fluorescein intensity. If the fluorescein intensity was not changed over time, we couldn't know which concentration was accurate. However, most measurement in this study was to calculate the relative change within a trial and it would always be zero for trials having constant intensity, no matter which concentration value we assigned.

Another potential issue with calculating fluorescein concentration from pixel intensity in our images was that the film thickness (meniscus depth) was needed during

the calculation, (Nichols, King-Smith et al. 2012; Braun, Gewecke et al. 2014) but was unknown. In this study, we estimated the meniscus depth from TMH, using ratio values from previous studies.(Zhou, Li et al. 2009; Ibrahim, Dogru et al. 2010; Bujak, Yiu et al. 2011; Qiu, Gong et al. 2011; Tittler, Bujak et al. 2011). Considering that these thickness values were only estimates, we calculated the effect of differing meniscus depths on fluorescein dye concentration, using values found in the literature and found little effect over film thicknesses when it was higher than 0.15mm (Figure 24). In this study, the average estimated meniscus depth was higher than that value (0.16± 0.04mm) during baseline and became much greater during tear secretion (0.21± 0.09mm). Thus, these potential errors in estimating film thickness should have induced minimal inaccuracies during the TMFC calculation.

In order to verify that our calculations of fluorescein dye concentration from intensity were valid, we developed an *in vitro* method to visualize known fluorescein dye concentrations with our slit lamp biomicroscope and digital camera system. The specialized technique of fluorophotometry is widely used for this type of calculation, (Webber, Jones et al. 1987; van Best, del Castillo Benitez et al. 1995; Tomlinson and Khanal 2005) so we wanted to ensure that our slit lamp biomicroscope system was sensitive enough to accurately capture changes in fluorescence intensity associated with changing dye concentration. Figure 26 shows our *in vitro* eye model data and demonstrates that the TMFC from both *in vitro* and experimental data fitted the mathematical model closely. Some of the experimental data curves slightly deviate away from the eye model curve, probably due difference in meniscus thickness between study subjects and eye model.

In this study, we analyzed the absolute value, maximum difference and slope of TMH and TMFC data within each trial to normalize the individual variation at the beginning of the trials. As discussed earlier, the TMH was highly variable and complicated by the opposing effects of blinking and tear secretion (Figure 20a). Understandably, the other TMH measures (TMH maximum difference and TMH slope) showed similar problems (Figures 20b and c).

In comparison, some of the calculated metrics of fluorescein dye concentration, TMFC, showed trends of increasing tear secretion with increasing stimulation, although variability remains high (Figure 20d-f), presumably due to the effects of blinking on tear secretion. The absolute value of TMFC was highly variable between subjects at the beginning of each trial (Figure 20d), which continued throughout the trial. This was probably due to different tear volumes between subjects, which produced a range of absolute dye concentrations within subjects. We normalized this variation by calculating the relative change over each trial (maximum difference, Figure 20e), rendering much clearer results. The TMFC maximum difference tended to increase all subjects with increasing air stimulation, although the amount of the increase differed. This may have been due to the variability in BR among subjects, causing differences in tear drainage, thus altering the TMFC maximum difference value. Interestingly, the TMFC slope did not show the same trends with increasing air stimulation in this pilot study (Figure 20f). One possible reason was that apparent tear secretion differed among trials changing its time and thus altering the slope value.

Thus, both the TMH and TMFC metrics were apparently affected by blinking, although TMFC, especially the maximum difference provided less confusing data. For this reason, we developed a novel metric, the IBI-TMFC slope. This measure is based on the slope of the change in calculated fluorescein dye concentration between blinks, and thus disengages blinking from tear secretion. This measure makes the assumption that changes in dye concentration are due to secretion, not tear drainage between blinks, which is thought to be minimal within 0.15sec after a blink (Rosengren 1972; Wilson and Merrill 1976) (Zhu and Chauhan 2005). Given the time measurements in this investigation, our study setup was very unlikely to catch that moment. As Figure 21 shows, the IBI-TMFC slope appears to provide less variable and more consistent data for tear secretion than other measures complicated by both tear secretion and blinking.

As we expected, tear secretion was increased with air intensity, presumably to protect the surface from potential damage.(Oyster 1999) Interestingly, the IBI-TMFC slopes were similar in the first few trials, and then increased dramatically. Thus, the IBI-TMFC slope, which was our best estimate of the tear secretion rate, was better fitted with an exponential function, rather than a linear one. One possible explanation was that different corneal receptors may have been activated with increasing corneal stimulation. When stimulus intensity was low, it is likely that only cold thermoreceptors were activated,(Murphy, Patel et al. 1996) and the tear secretion remained close to its basal level.(Parra, Madrid et al. 2010) As stimulus intensity increased, it is probable that polymodal and mechanical nociceptors were also activated, inducing reflex tearing(Belmonte, Aracil et al. 2004; Parra, Madrid et al. 2010); thus, significantly increasing the IBI-TMFC slope. This probable masking effect that occurred with

activation of the mechano- and polymodal nociceptors may have accounted for the appearance of an exponential increase in tearing with increasing stimulation. In contrast, Situ and Simpson found a linear increase in tear secretion with increasing corneal stimulation, rather than exponential. However, the stimuli used in that study differed from ours and may have activated mostly mechano- or/and polymodal nociceptors. Thus, their results may fit well in the second half of our curve where the exponential increase occurs. (Situ and Simpson 2010)

In our previous study, we found there was a linear correlation between air intensity and IBI duration.(Wu, Begley et al. 2014) In this Chapter (#4), we found the similar results, even though there was a relatively high individual variation between subjects. The increasing BR with stimulation presumably occurred to protect the ocular surface by decreasing the ocular exposure time and, with tearing, rewetting the surface more frequently.(Rosengren 1972; Schein, Tielsch et al. 1997; Acosta, Gallar et al. 1999; Alex, Edwards et al. 2013) The reasons for the high individual variation in IBI in this Chapter might be due to the relatively air stimulus and used in this study compared to our previous study. Also, the sample size in this pilot study was relatively low and BR is highly variable, even within healthy subjects. (Carney and Hill 1982; Doughty and Naase 2006)

The differing time points of the blink and tearing response was unexpected. The reason for the average five seconds delay, in new tear secretion, is unclear. Presumably, the initial pathway for signaling required similar timing for both responses, although it is possible that different neuron subclasses at the ocular surface were involved. (Stapleton, Marfurt et al. 2013) Likewise, transmission times to the muscles controlling the blink and

glandular secretion of tears were likely to be relatively similar, at least compared to the rather large 5 second lag between the two responses noted in this study. Thus, the most plausible reasons for the delay in tear secretion are the time required for tear production and/or the time required for the tears to appear in the lower meniscus following secretion. According to the literature, "aqueous tears are secreted into the supero-lateral fornix by the main and palpebral portion of the lacrimal gland and into the upper fornix by the accessory lacrimal glands."(Gaffney, Tiffany et al. 2010) Thus, the delay in tearing might represent the time needed for tears to flow from the fornices to the meniscus following secretion.

In this study, the BR decreased over time during ocular surface stimulation, almost back to baseline levels. This apparent adaptation to the stimulus may be related to the neural characteristics of corneal receptors. Although the exact nature of stimulation on the ocular surface was unknown, it was very possible that the air flow induced a mechanical force on the surface, as well as cooling and increased hyperosmolarity in the tear film. (Murphy, Patel et al. 1996; Belmonte, Acosta et al. 1999) Thus, as discussed above, several types of corneal receptors could have been activated by the stimulus used in this study and most show adaptation to a stimulus. Corneal mechano-nociceptors are A-δ neurons are fast conducting, which could have contributed to the almost immediate reflex blink response. (Belmonte, Acosta et al. 2004) This type of neuron is fast-adapting and encodes the presence and intensity of the stimulus, and also its duration.(Belmonte, Aracil et al. 2004) Several studies have shown that mechano-nociceptors adapt rapidly to mechanical stimuli presented to the human cornea, (Chen, Feng et al. 2010; Chen and Simpson 2011) and thus may have contributed to the blink adaptation found in this study.

Decreasing neural firing rates with stimulation have also been found in cold thermoreceptor and polymodal nociceptors when cooling and acid stimulation was applied to the cornea. (Gallar, Pozo et al. 1993; Chen, Gallar et al. 1995; Hirata and Meng 2010; Parra, Madrid et al. 2010) Right after stimulation, there was a transient increase in nerve terminal impulses that lasted for about 10-30 seconds (dynamic period) and then dropped to basal levels (static period). (Parra, Madrid et al. 2010; Cardona, Garcia et al. 2011) Thus, all types of corneal neurons can show adaptation to a stimulus and thus may have been involved in the adaptation of blink response found in this study.

Along with blinking, tear secretion also appeared to show adaptation to the pneumatic stimulus used in this study. Holly and co-workers found a large increase in tear secretion after applying a mild trigeminal stimulus, and then the secretion rate was diminished exponentially until it stabilized at a low rate.(Holly, Lamberts et al. 1982; Holly, LauKaitis et al. 1984) However, although many have studied the tear secretion response, most did not monitor tear secretion over time, (Belmonte, Aracil et al. 2004; Situ and Simpson 2010; Koh, Tung et al. 2012; Alex, Edwards et al. 2013) making it difficult to compare our results to previous findings.

Since both blink and tear receive the inputs from the ocular surface, their correlation was also tested. The overall correlation was moderate, highlighting their common origin from the ocular surface. However, the correlation was poor within subject, which might be due to the response difference between tearing and blinking. Sometimes, there was a big blink increment, but not much tear secretion change, or vice versa. Although both blink and tear secretion were increased with air stimulation, the amplitude of their responses were different, leading to a poor correlation within subject.

There are several limitations in this study that may have affected our results. Fluorescein dye was instilled multiple times for improved visualization of the lower tear meniscus. This may have produced ocular irritation, affecting the normal tear secretion rate.(Mishima, Gasset et al. 1966) In addition, subjects were asked to look slightly down to better view the meniscus against the cornea for purposes of image processing, which may have affected blink patterns.(Cho, Sheng et al. 2000) However, all the trials were performed under the same conditions, and comparisons were made to baseline conditions, so these effects should be minimized. Moreover, the evaporation may also have played a role, but its effect is likely to be minimal given the high tear volume within the meniscus and its relatively small area of exposure. In addition, our data do not show evidence of measurable evaporation, by either TMH or TMFC measures (see the long IBI periods in Figure 16d-e) and the high BR with stimulation should also have acted to decrease evaporation.

This study was designed to study the timing and extent of the two main reflexive mechanisms available to the ocular surface in response to adverse stimulation. We developed novel metrics, TMFC, to allow the study of both the blink and tearing response air stimulation of the cornea. Not surprisingly, we found that both blinking (Wu, Begley et al. 2014) and tearing increased (Situ and Simpson 2010) with increasing ocular surface stimulation, presumably to protect the surface. However, an unexpected finding was the time lag for newly secreted tears five seconds after the blink response, on average. The reason for this lag between the two responses is unknown and has not been reported previously to our knowledge. From an evolutionary perspective, it be due to the need for a very rapid blink reflex (Evinger and Manning 1993) to protect the eye from projectiles

or foreign objects, (Levin and Kaufman 2011) whereas the slight delay in reflex tearing may be fully adequate for washing out particles or other debris. Another unexpected finding was the apparent adaptation of both blinking (Evinger and Manning 1988) and tearing during the relatively short stimulus time used in this study. This appears counter-productive from the standpoint of maintaining an adequately wetted ocular surface with air stimulation, although, perhaps the initial blinking and tearing response laid down a robust tear film able to withstand the air stimulus so that interruption of vision was minimized. The reasons presented here for some of our findings are speculative and require further study to more fully understand the dynamics and underlying neural mechanisms of blinking and tear secretion in response to ocular surface stimulation.

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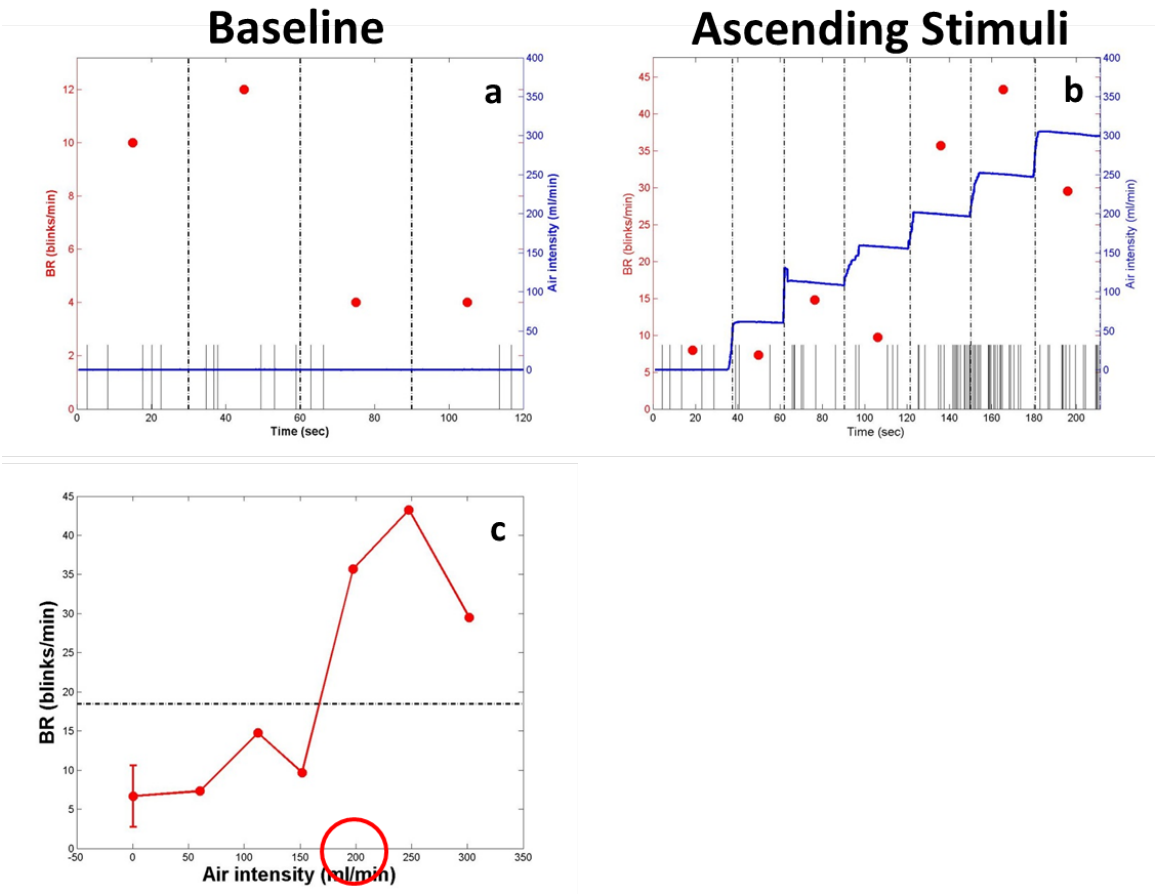

Figure 1: Individual example for setting the stimulus range. a) Blink response without stimulation in four minutes (baseline). The small vertical bars denote the timing of blinks, and the blue line indicates the air intensity over time. The red dots represent the blink rate (BR) within each minute. b) Blink response with increasing air stimulation. The red dots are the BRs under different levels of air stimulation. c) Determine the air stimulus that triggering high BR. BR is plotted as a function of air intensity. The dot and error bar at zero x axis are the mean and standard deviation of BR during baseline. The dash line represents the three standard deviations above the mean BR during the baseline. The red circle shows the air intensity that triggering high BR in this subject.

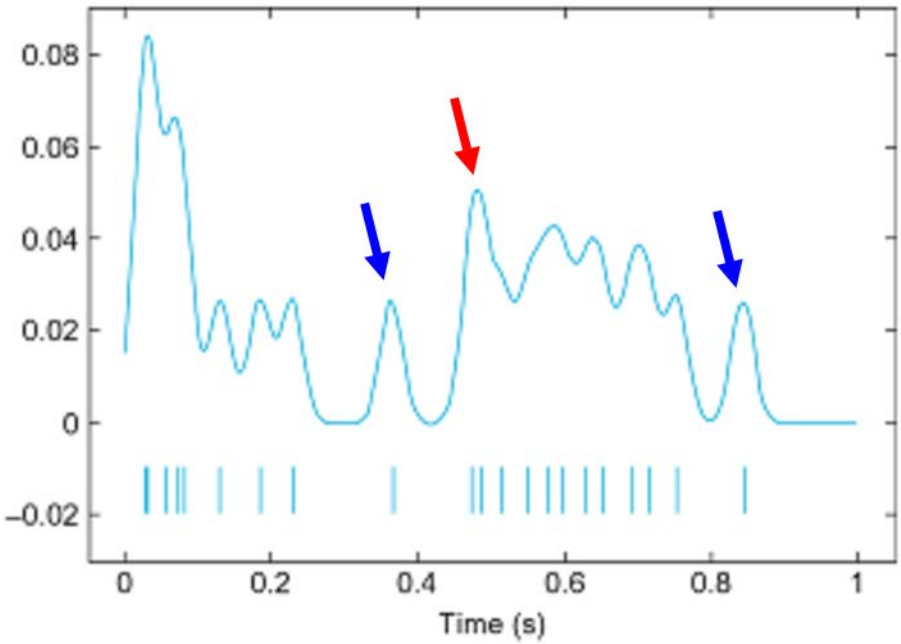

Figure 2: An example of spike density analysis on neural activity.(Wallisch, Lusignan et al.) The lower blue bars represent the original neuron spike activity over time. The upper blue curve represents the final integrated curve by replacing each spike with a normal curve. The blue arrow shows that a low peak is created when low frequency spike occurrence. The ted arrow shows that a high peak is created when high frequency spikes occurrence.

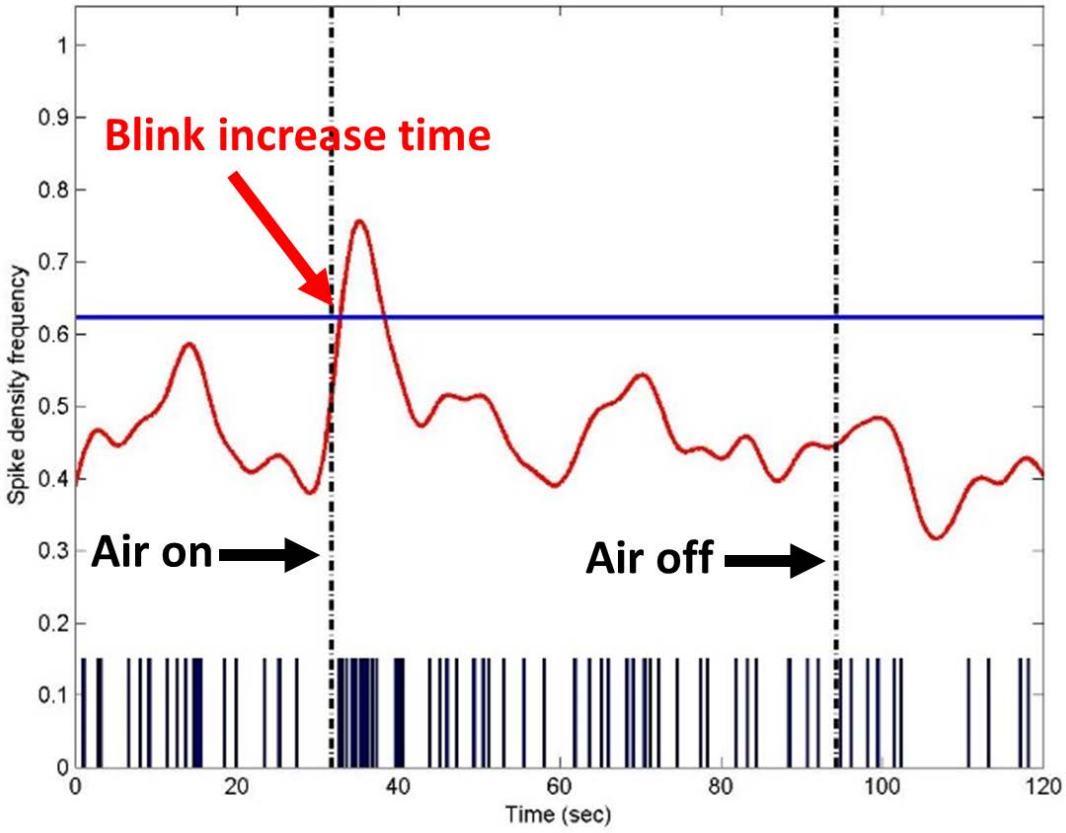

Figure 3: Individual example of determine the blink increase threshold. The small vertical bars denote the timing of blinks, and the red curve is the final output after spike density analysis. The vertical dash lines show the time of air stimulus on and off. . The horizontal blue line is the three standard deviations above the mean value of the red curve before the air stimulus was on.

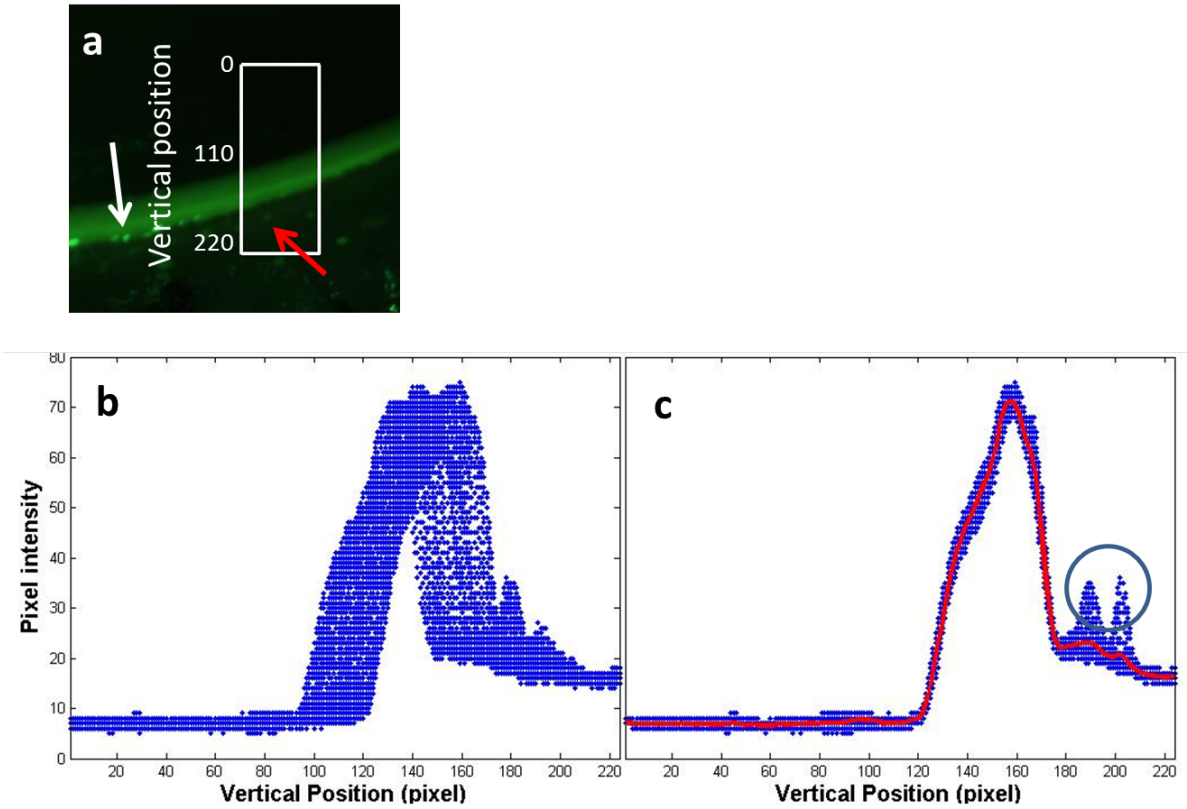

Figure 4: Individual example of single image analysis for lower tear meniscus. a) raw color image of the lower meniscus. The white box shows the region of interest (ROI), which will be analyzed further. The red arrow shows some fluorescein spilled on the lower eyelid, and the white arrow shows the bright reflection from the silt lamp. b) pixel intensity as the function of vertical position in each column within ROI. c) the final curve calculated from each image. Red curve: the average intensity from all the columns, circle: image noise from fluorescein spilled on the lower lid

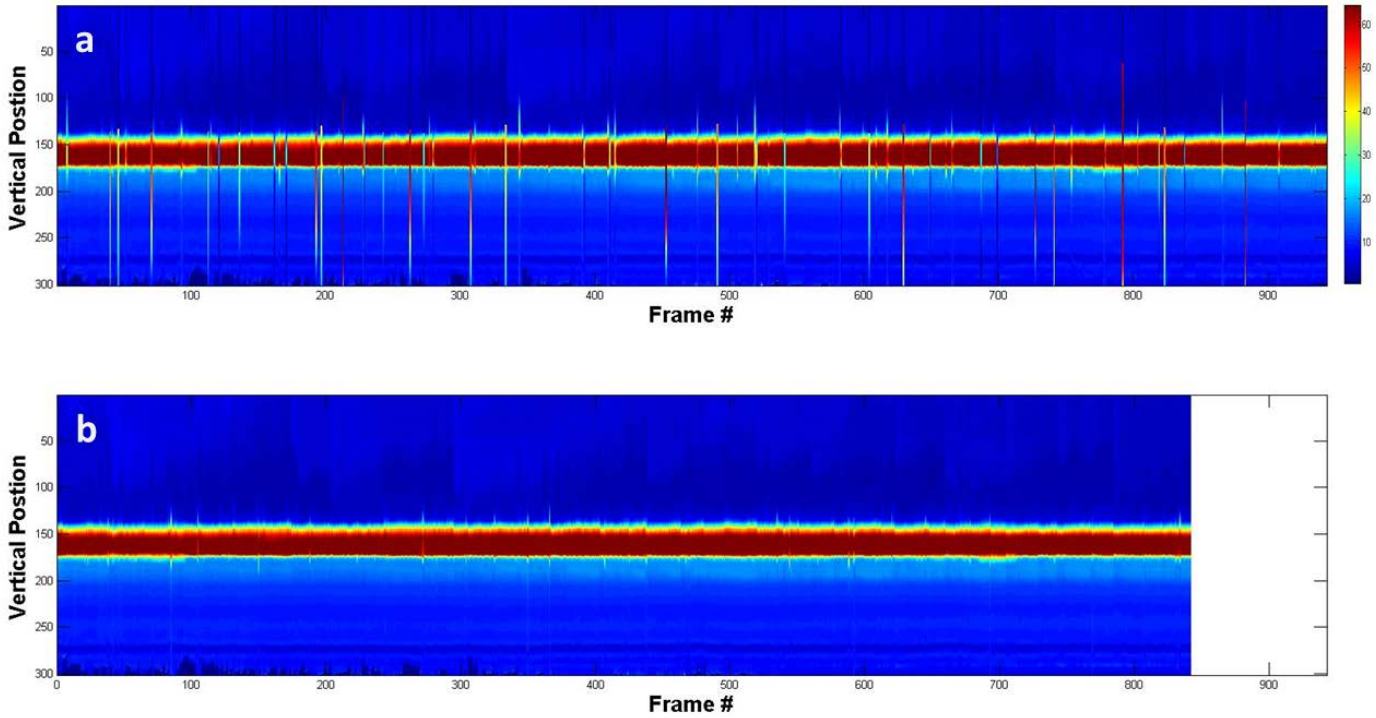

Figure 5: Individual example of taking off the frames with blinking. a) color map generated from a raw trial. Color bar represents the pixel intensity. b) color map generated from a trial after taking off the blink frames.

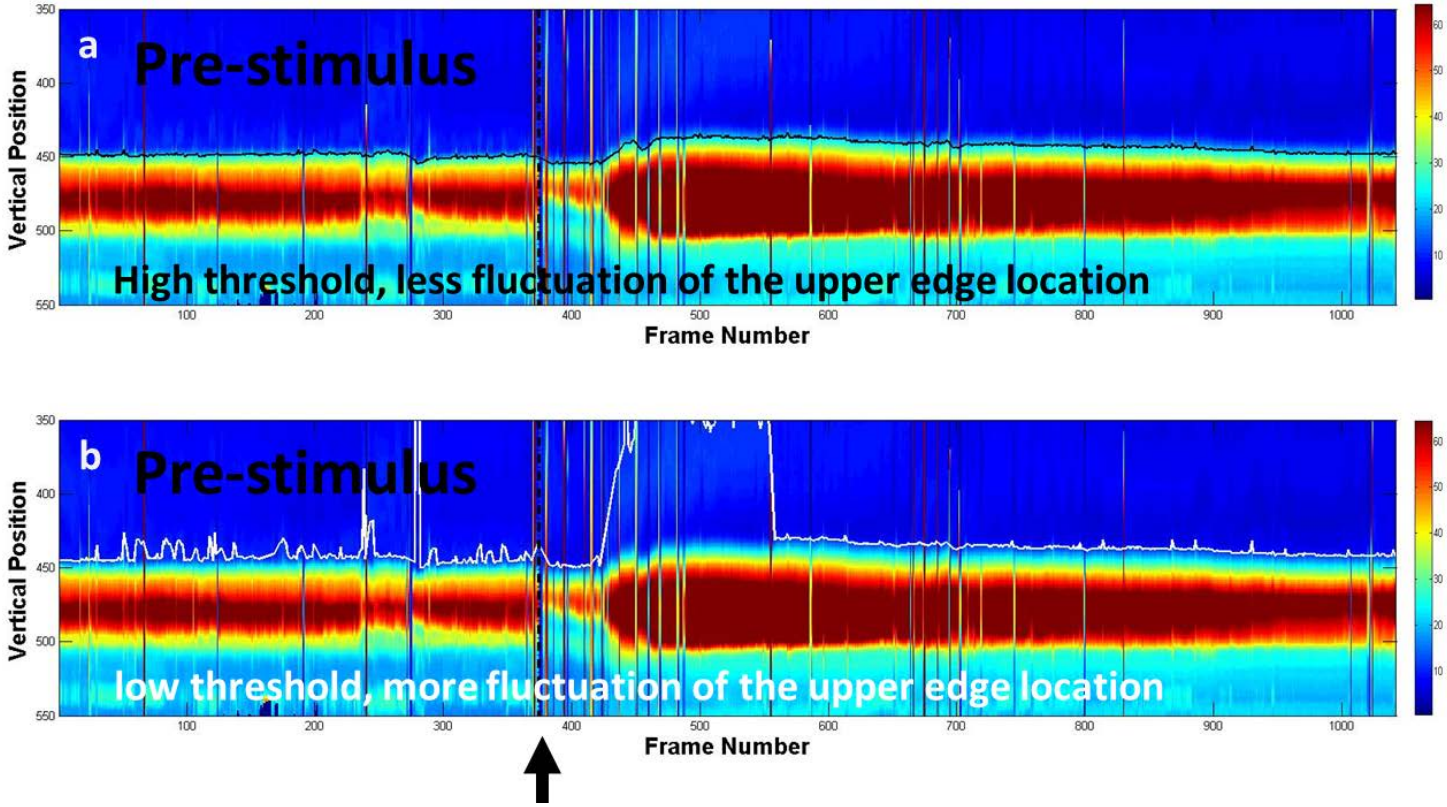

Figure 6: Individual example of using different thresholds to find the upper edge of the lower meniscus. a) using a high threshold, b) using a low threshold. Black arrow shows the time for air stimulus was on.

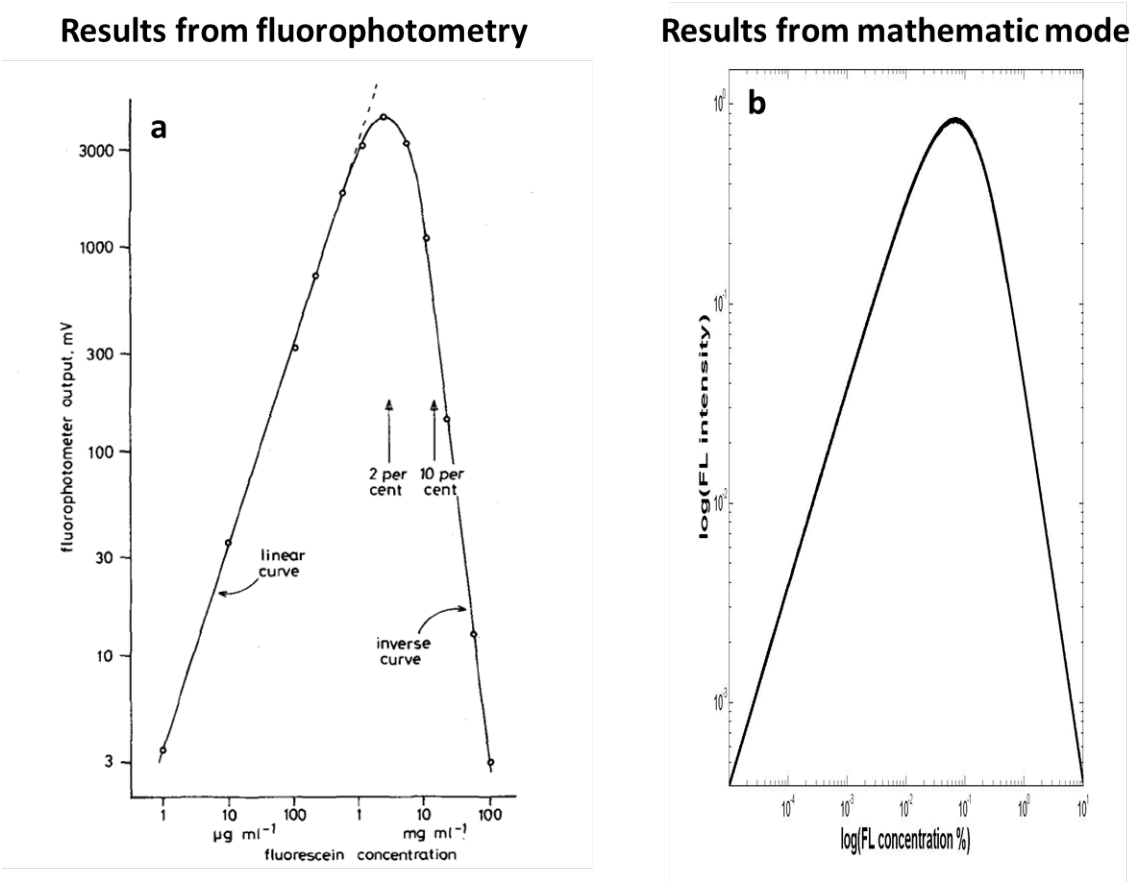

Figure 7: the correlation between fluorescein concentration and intensity. a) result from Fluorophotometry,(Webber and Jones 1986) b) result from mathematical model (Nichols, King-Smith et al. 2012) The film thickness was assuming as 5µm in both figures.

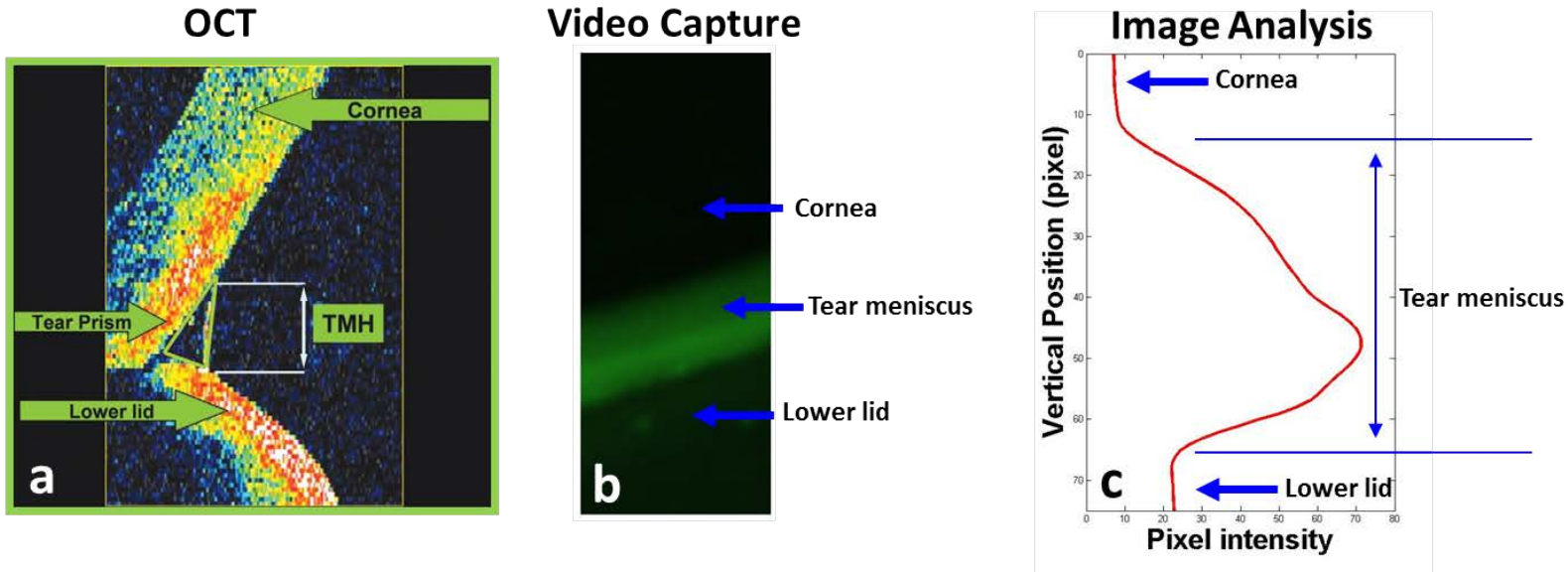

Figure 8: Imaging the lower meniscus with different techniques. a) meniscus was monitored by optical coherence tomography (OCT).(Srinivasan, Chan et al. 2007) b) meniscus was monitored under silt lamp. c) image analysis of the fluorescein intensity across the lower meniscus.

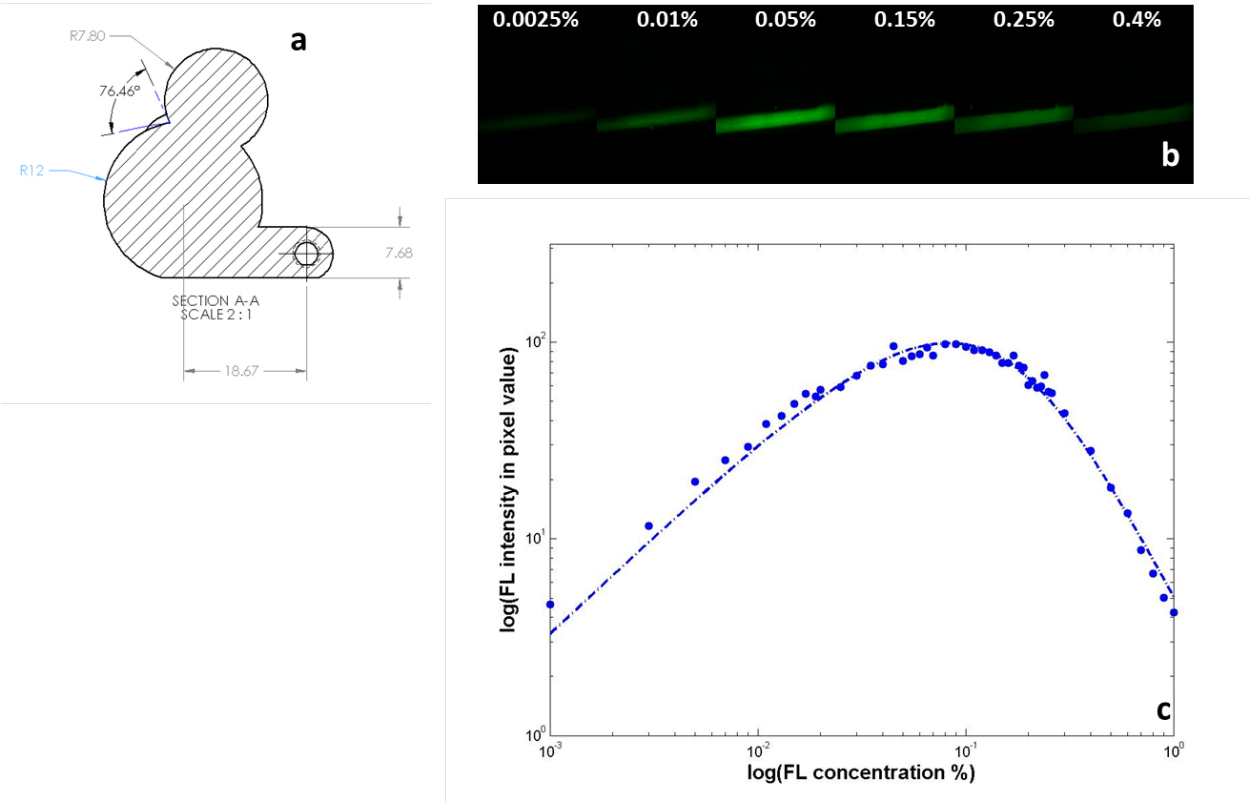

Figure 9: the in vivo eye model. a) Schematic figure of the eye model. b) images captured from the silt lamp with known fluorescein concentration instilled on the eye model. c) correlation between fluorescein concentration and intensity collected from the eye model. Data was fitted with the mathematical model (blue line).

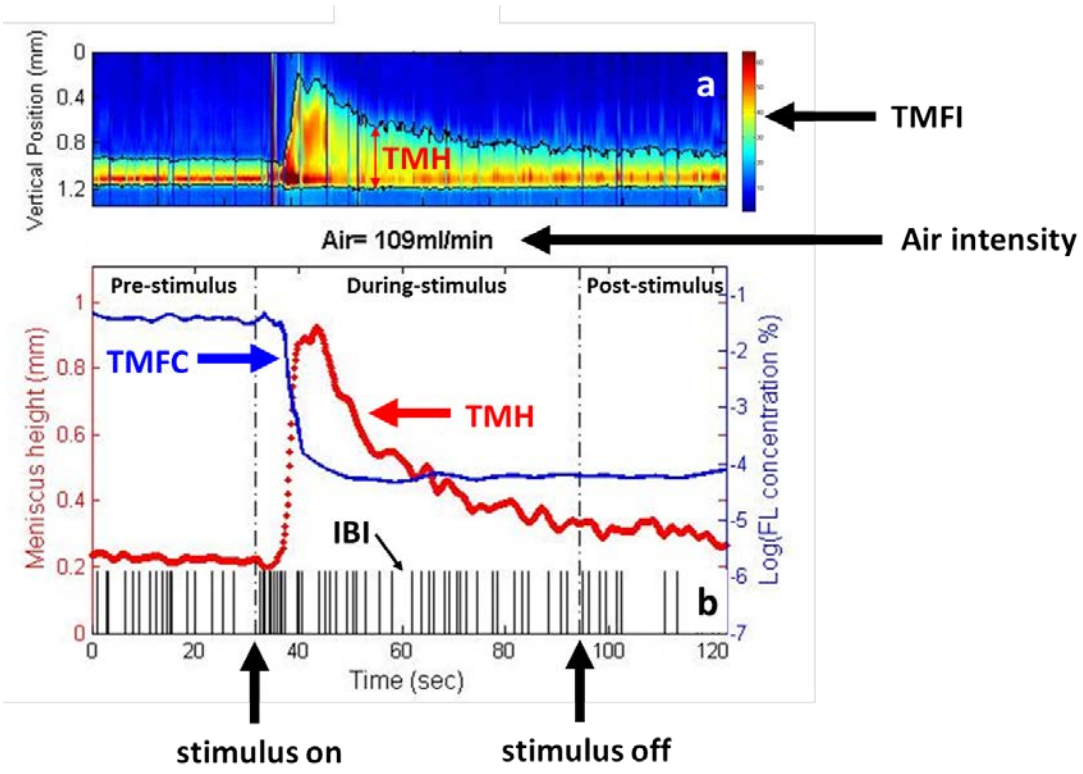

Figure 10: Individual example of the raw data analyzed from each trial. a) color map created from each trial. TMFI: tear meniscus fluorescein intensity b) calculated parameters over time. The red line indicates the tear meniscus height (TMH), the blue line indicated the tear meniscus fluorescein concentration (TMFC), the small vertical bars denote the timing of blinks and the black dash lines represent the timing of air stimulus on and off. The average air intensity during the central 1 minute is shown between the color map and parameter figure.

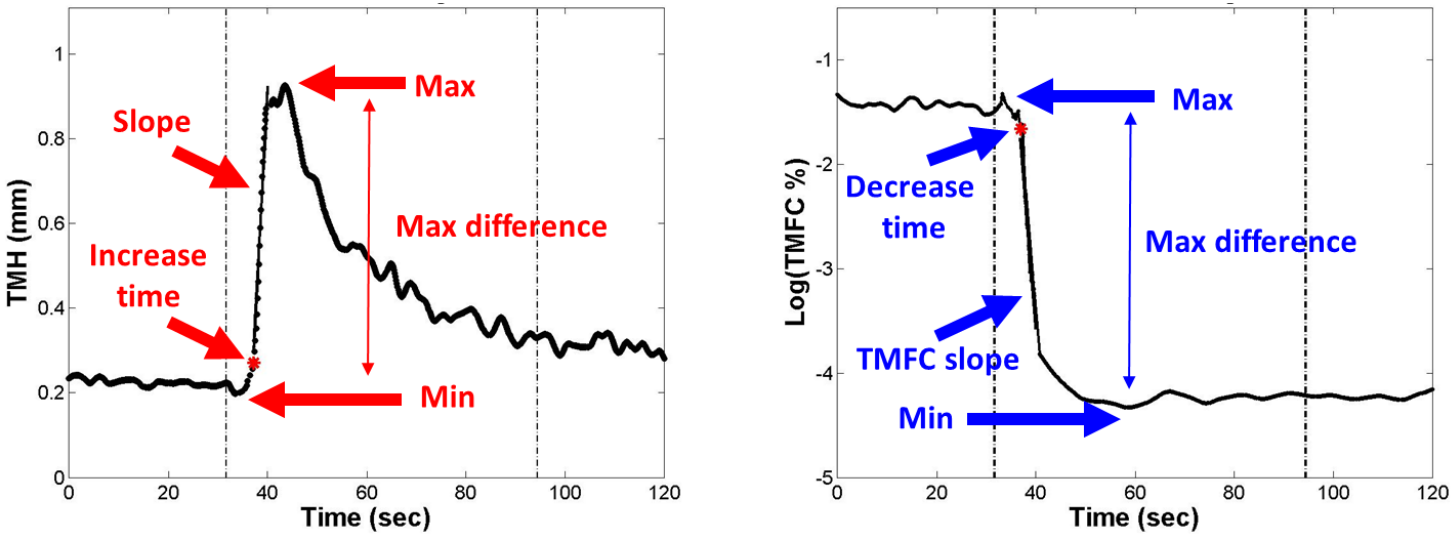

Figure 11: Further calculation on TMH and TMFC in each trial. Maximum difference and slope are calculated on TMH and TMFC, as well as the responding time. TMH: tear meniscus height. TMFC: tear meniscus fluorescein concentration.

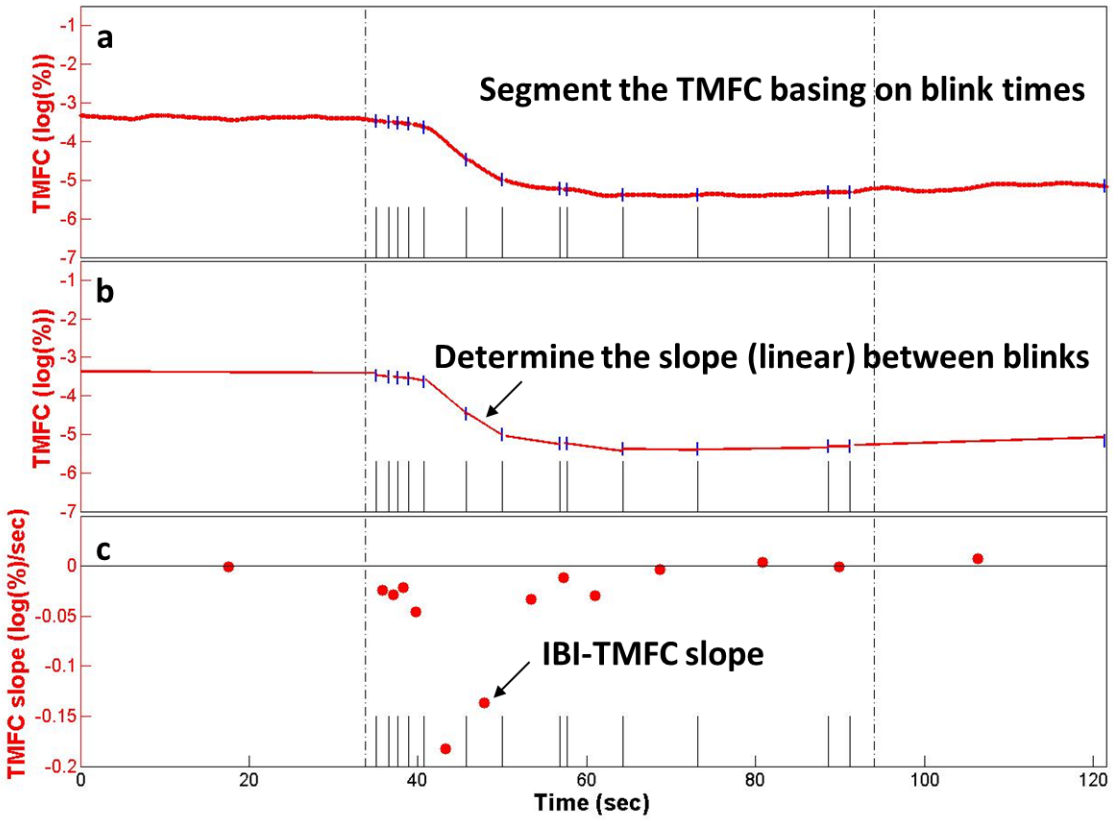

Figure 12: Individual example of the calculation of IBI-TMFC slope from a trial. a) the fluorescein concentration data was segmented basing on blink time. b) a linear regression line was fitted within each segment. 3) the slope was calculated within each segment. If there was no or minimal tear secretion, the slope would be close to zero. If there was a big tear secretion, the slope would be more negative.

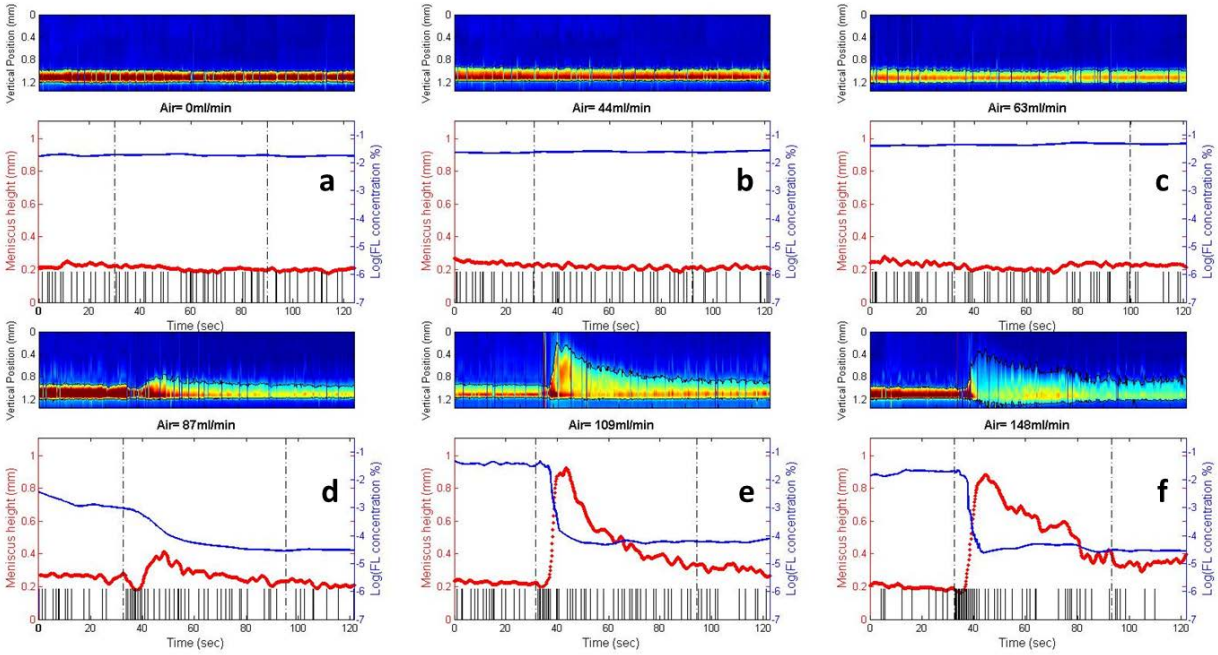

Figure 13: Blinking and tear secretion responses (Subject 1) to six levels of air stimulation from 0 to 148ml/min (a-f). Within each individual graph, the red line indicates the tear meniscus height, the blue line indicated the tear meniscus fluorescein concentration, the small vertical bars denote the timing of blinks and the black dash lines represent the timing of air stimulus on and off. The corresponding color map is also shown above the raw data for better visualization on tear meniscus. The average air intensity during the central 1 minute is shown between the color map and raw data figure for each trial.

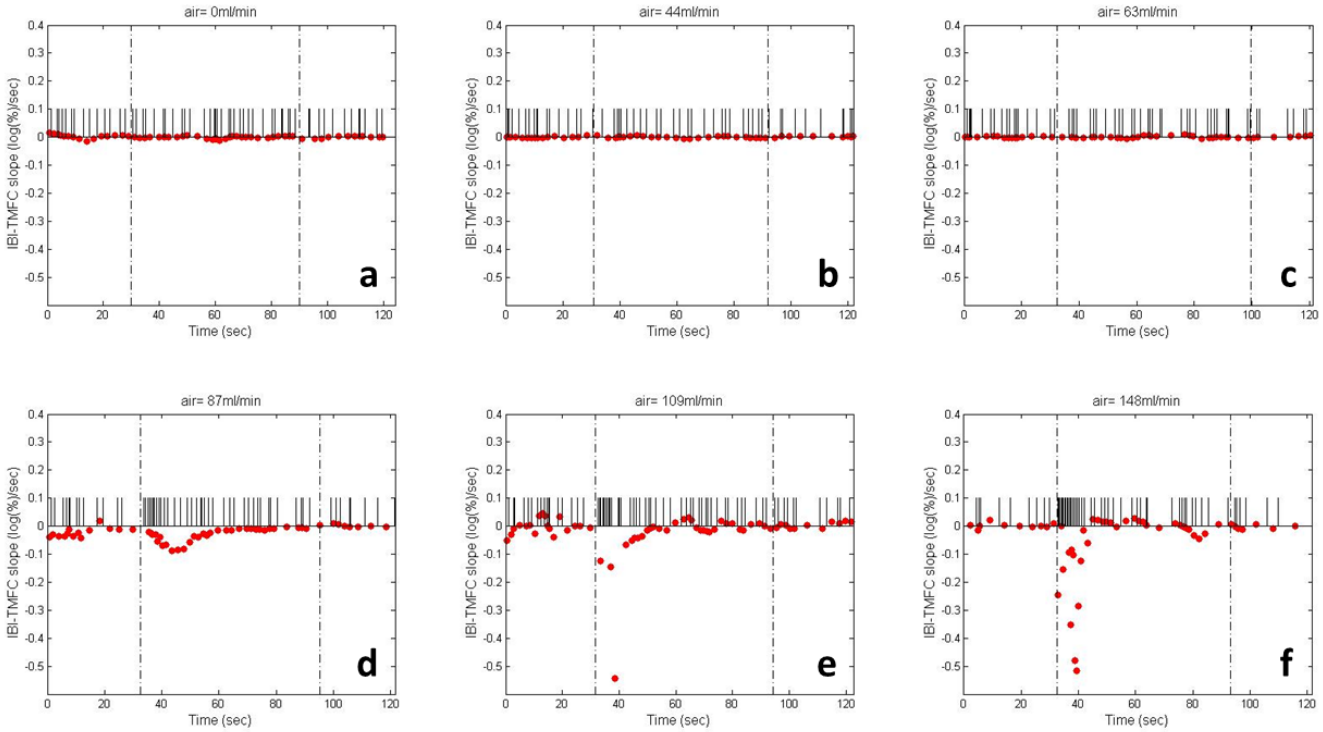

Figure 14: The IBI-TMFC slope responses (Subject 1) to six levels of air stimulation from 0 to 148ml/min (a-f). Within each individual graph, the red dot represents the IBI-TMFC slope, the small vertical bars denote the timing of blinks and the black dash lines represent the timing of air stimulus on and off.

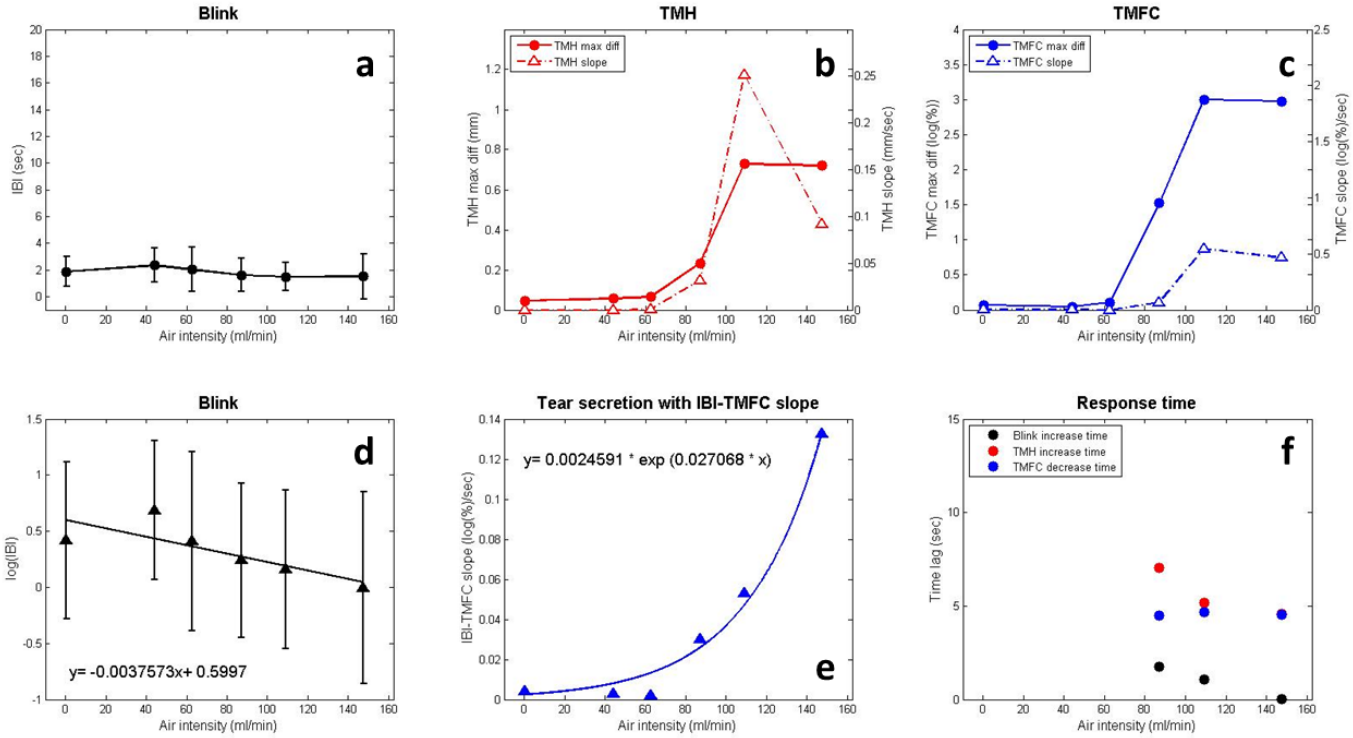

Figure 15: All the data summarized from Subject 1 and are plot as a function of air intensity. a) mean and standard deviation of IBI. b) TMH maximum difference and slope. c) TMFC maximum difference and slope. d) log transformation of mean IBI. e) average IBI-TMFC slope. An exponential function was fitted and the equation is shown at top left. f) responding time of blink and tear secretion.

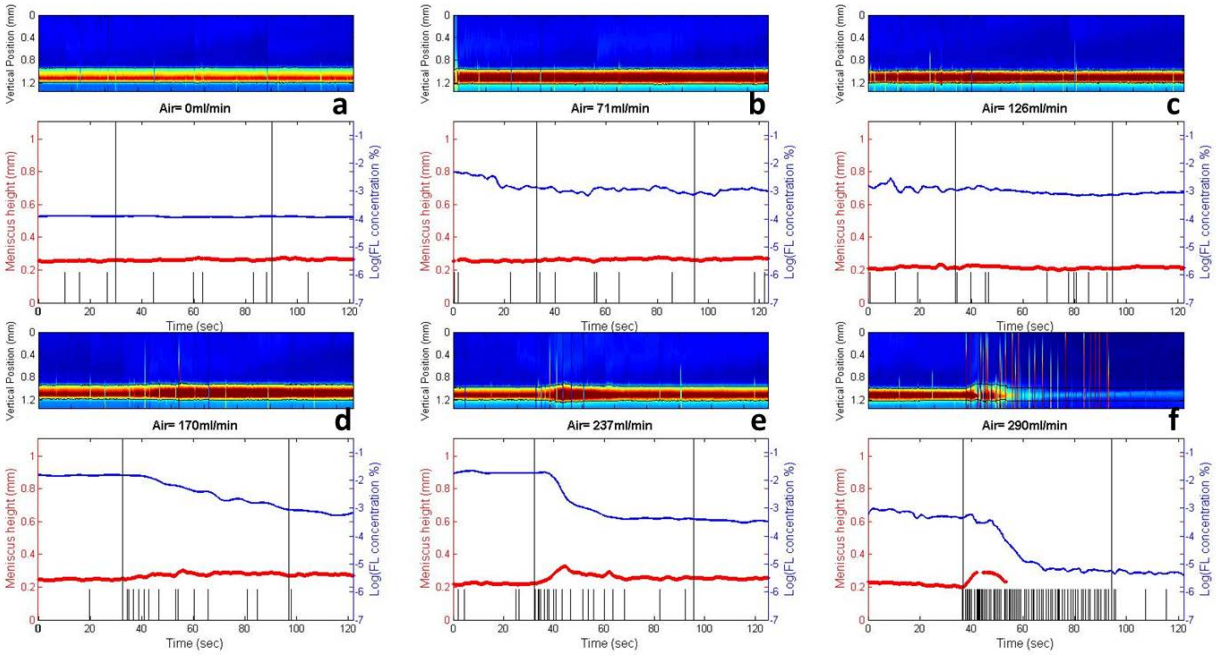

Figure 16: Blinking and tear secretion responses (Subject 2) to six levels of air stimulation from 0 to 290ml/min (a-f). The panels are formatted identically to Figure 13. Note the lack of change in TMH in panel f when rapid blinking occurred.

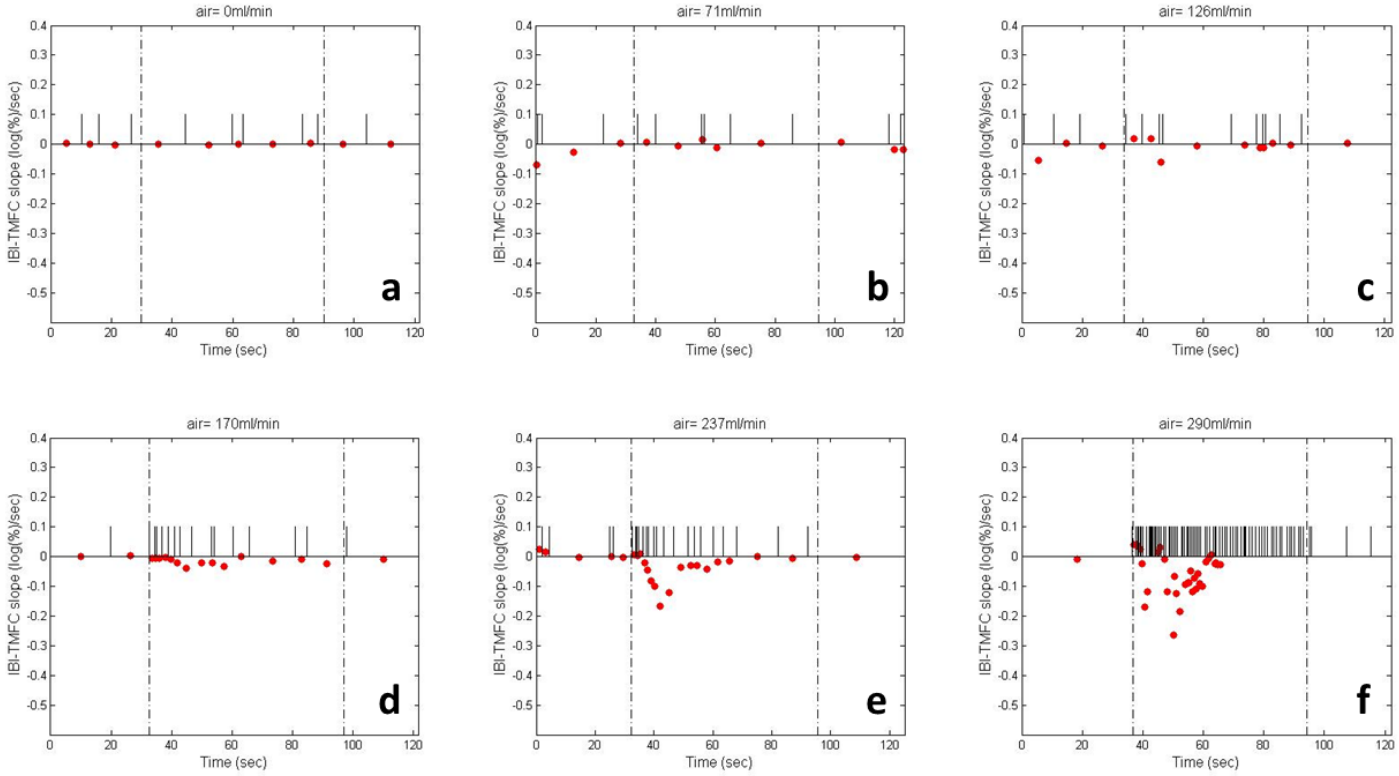

Figure 17: The IBI-TMFC slope responses (Subject 2) to six levels of air stimulation from 0 to 290ml/min (a-f). Within each individual graph, the red dot represents the IBI-TMFC slope, the small vertical bars denote the timing of blinks and the black dash lines represent the timing of air stimulus on and off.

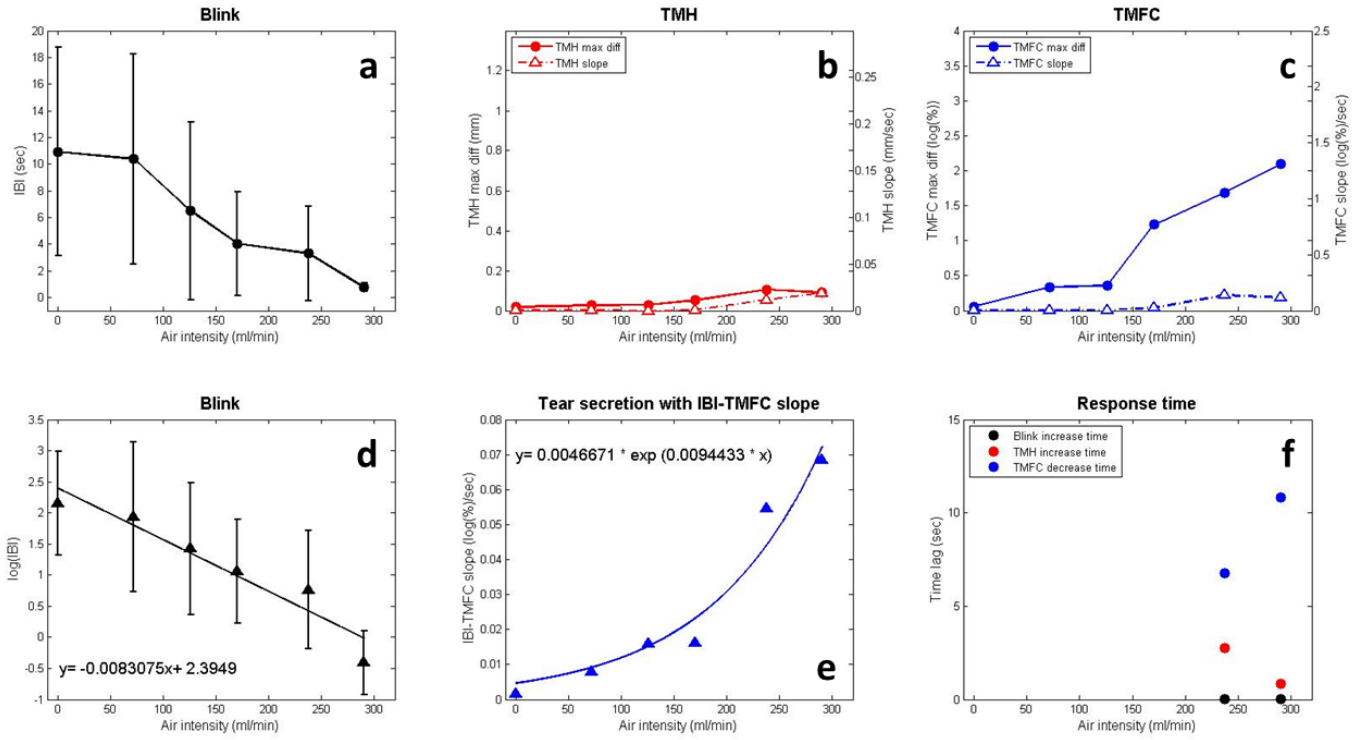

Figure 18: All the data summarized from Subject 2 and are plot as a function of air intensity. a) mean and standard deviation of IBI. b) TMH maximum difference and slope. c) TMFC maximum difference and slope. d) log transformation of mean IBI. e) average IBI-TMFC slope. An exponential function was fitted and the equation is shown at top left. f) responding time of blink and tear secretion.

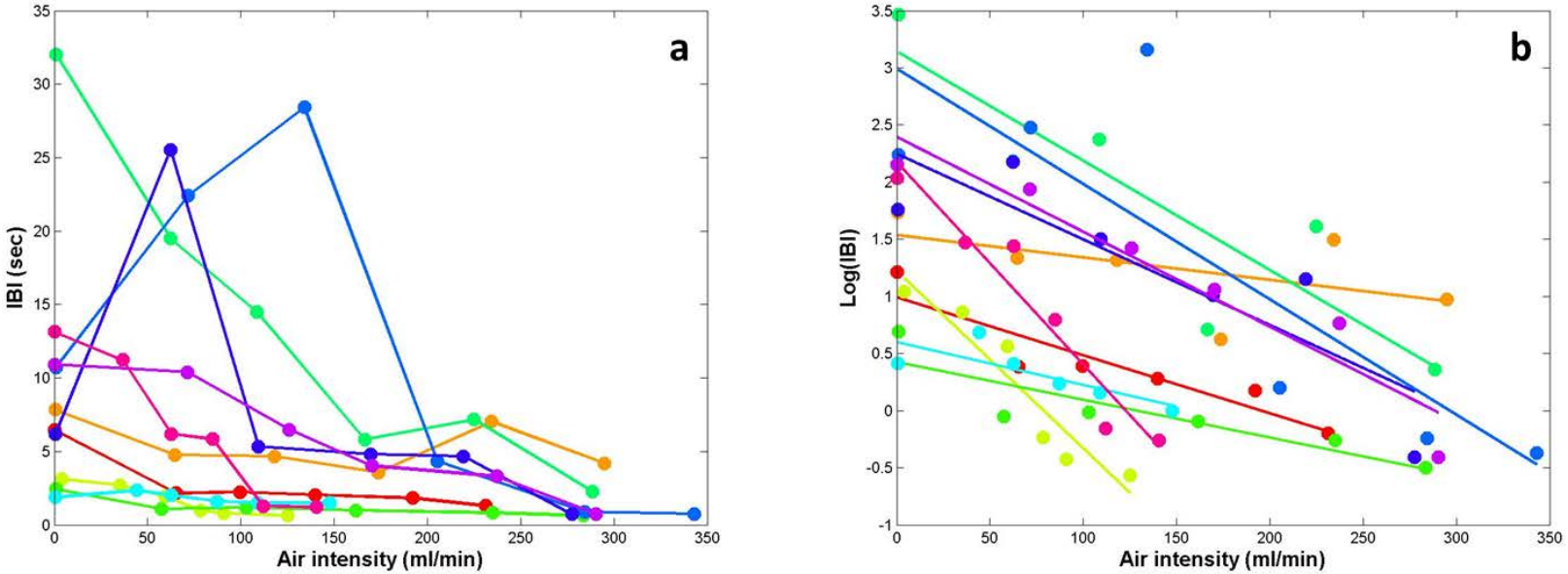

Figure 19: Blink response for all subjects. a) IBI under different air stimulation. b) Log of IBI under different air stimulation with linear regression line fitted within each subject.

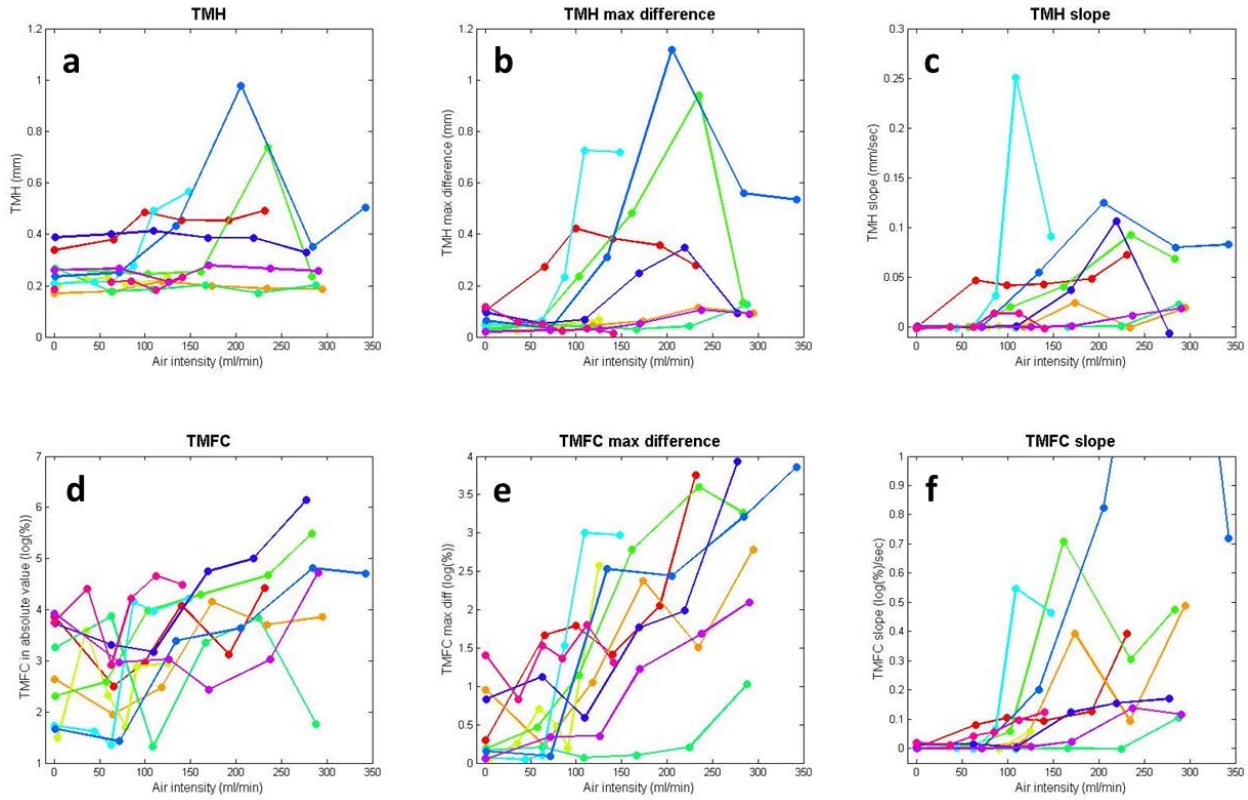

Figure 20: Aggregate data of tear meniscus for all the subjects as the function of air intensity. TMH: tear meniscus height; TMFC: tear meniscus fluorescein concentration.

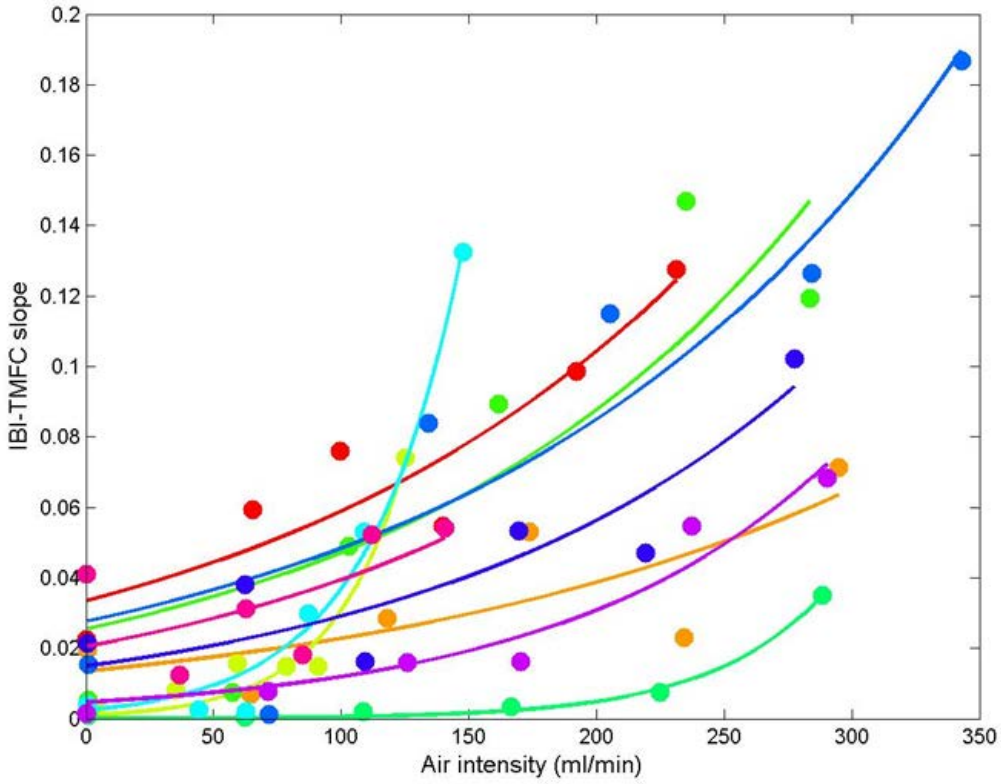

Figure 21: aggregate data of IBI-TMFC slope for all the subjects. Each point is the average IBI-TMFC slope value within a trial.

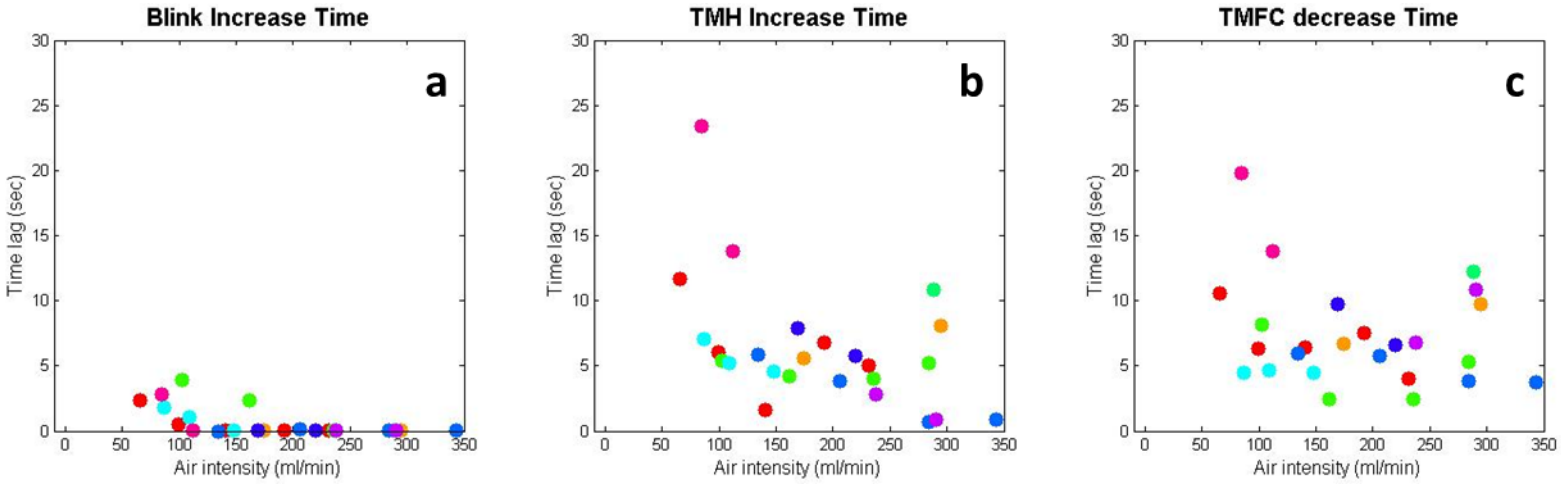

Figure 22: Aggregate data of responding time for all the subjects.

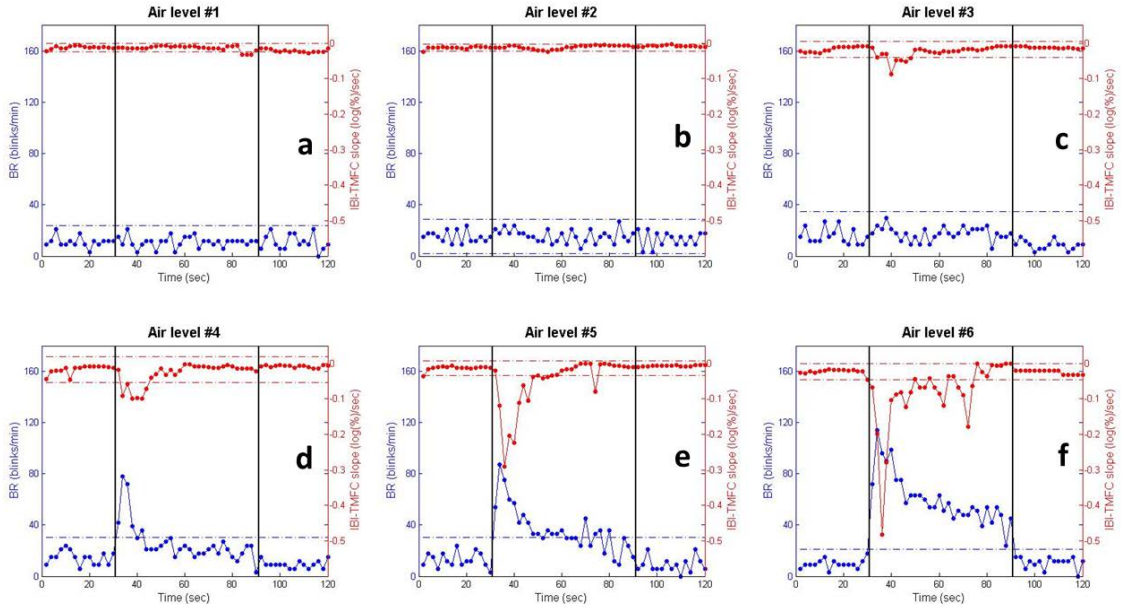

Figure 23: Aggregate data for blink rate and IBI-TMFC slope for all the subjects. Each trial is segmented into 60 windows that each window is about 2 seconds. The average blink rate (BR) and IBI-TMFC slope were calculated in each window and the mean values from all the subjects are shown here. Within each individual plot, each blue dot is the average blink rate (BR), the red dot is the average IBI-TMFC slope, the black vertical lines are the timing of air stimulus on and off. The blue dash line is the 3 standard deviations above the mean BR value during the pre-stimulus period. The red dash lines are the 3 standard deviation from the mean IBI-TMFC slope during the pre-stimulus period.

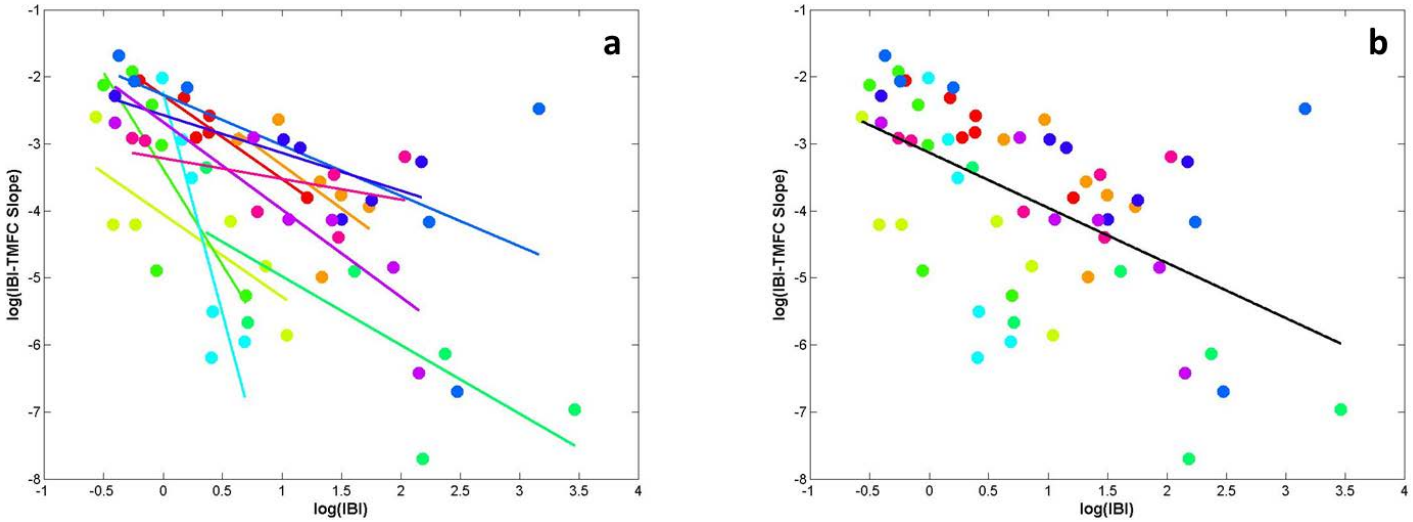

Figure 24: Correlation between IBI and IBI-TMFC slope in log space. a) a linear regression line was fitted within each subject. b) a linear regression line was fitted for all the subjects from all the trails. (Pearson r= -0.556, $p<0.0001$)

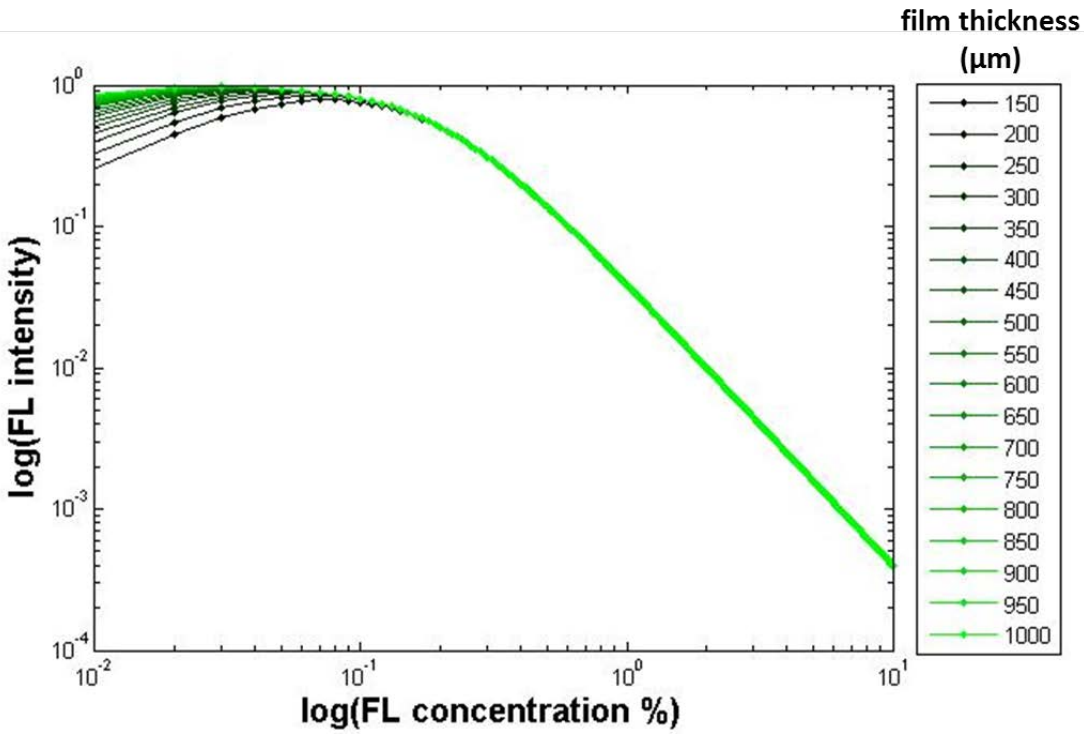

Figure 25: Correlation between fluorescein concentration and intensity with different film thickness. (Nichols, King-Smith et al. 2012)

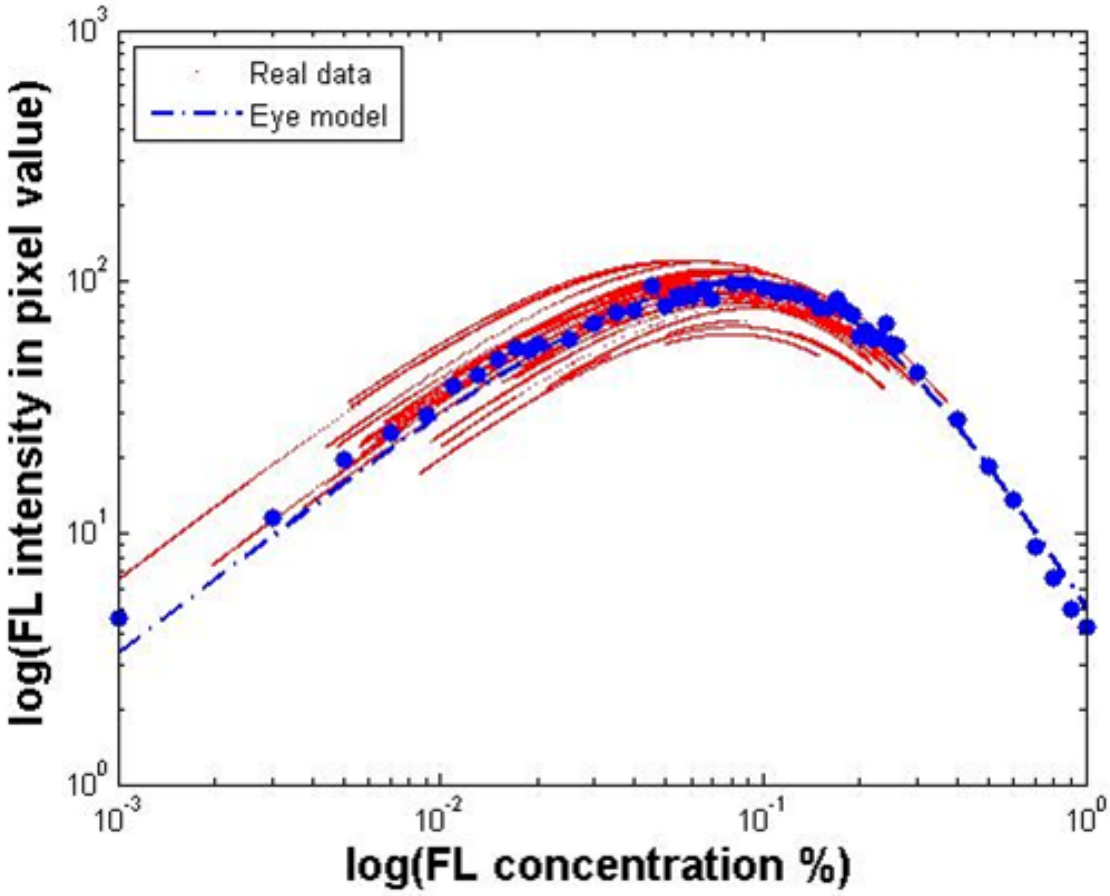

Figure 26: Compare the calculated TMFC from real subjects with the data collected from eye model. The red lines are the calculated TMFC from all the subject all the trials. The blue dots are the data from eye model and were fitted with the mathematical model. (Nichols, King-Smith et al. 2012)

CHAPTER V

SUMMARY OF THESIS

Dry eye is a common disease that affects millions in the United States (Schaumberg, Sullivan et al. 2003; Schaumberg, Dana et al. 2009) and worldwide.(McCarty, Bansal et al. 1998; Uchino, Nishiwaki et al. 2011) It is considered a multifactorial condition driven by tear film instability and hyperosmolarity with possible damage to the ocular surface.(2007) In the normal condition, the ocular surface is mainly protected by blinking and tearing.(Levin and Kaufman 2011) During a blink, the upper lid wipes the ocular surface, spreading the newly secreted tears over the ocular surface.(Levin and Kaufman 2011) Despite the importance of blinking and tearing in maintaining a healthy ocular surface and thus avoiding the dry eye condition, the dynamics of these two responses, and their interaction with each other, remain unclear.

In the chapter 2, we first studied the blink response under a mild surface stimulation, while varying the concentration on a visual task. Basing on previous studies, the blink frequency was increased with ocular surface stimulation that a range of stimuli were used.(Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999; Toda, Asano-Kato et al. 2001; Borges, Garcia et al. 2010; Jansen, Begley et al. 2010) Many of the studies investigating the effect of ocular surface on human blink rate have involved relatively dramatic or sizeable changes in ocular surface inputs, such as abrogating ocular surface

sensation with anesthetic,(Naase, Doughty et al. 2005; Borges, Garcia et al. 2010) damage to corneal nerve after LASIK surgery,(Toda, Asano-Kato et al. 2001) or placing contact lens, which could be considered an irritant to the ocular surface.(Jansen, Begley et al. 2010) However, there was no study measured the blinking while applying a low level stimulus on the ocular surface, which is similar as the early period of dry eye condition.

Traditionally, blink frequency, including blink rate and interblink interval,(Bentivoglio, Bressman et al. 1997; Nakamori, Odawara et al. 1997; Belmonte, Acosta et al. 1999; Schlote, Kadner et al. 2004; Naase, Doughty et al. 2005) were measured to quantify the blink activity. Latest studies have shown that the quality of the blink, such as blink amplitude, also plays an important role in maintaining tear film integrity, thus, might be altered under different conditions.(Harrison, Begley et al. 2008; Cardona, Garcia et al. 2011; Hirota, Uozato et al. 2013) In this study, I measured the blink frequency, as well as the detailed blink parameters, including amplitude, duration and velocity, thus, monitoring the blink in more aspects.

In the Chapter 2, we found that the cognitive concentration and ocular surface had opposite effects on blink frequency and duration. High concentration on a visual task was associated with a decrease in the blink frequency and duration, presumably to minimize interruption of vision by the eyelids.(Schlote, Kadner et al. 2004; Himebaugh, Begley et al. 2009; Jansen, Begley et al. 2010; Cardona, Garcia et al. 2011) Ocular surface stimulation tended to increase the blink rate and duration, presumably to protect surface from irritation.(Nakamori, Odawara et al. 1997; Belmonte, Acosta et al. 1999)

Blink amplitude was highly variable between and within subjects, which might obscure the difference in blinking between the conditions tested in this study.(Himebaugh, Begley et al. 2009; Jansen, Begley et al. 2010; Cardona, Garcia et al. 2011) In addition, no significant difference was found in blink velocity between conditions, which may be due to its high correlation with blink amplitude.(Evinger, Manning et al. 1991) In this study, we found a similar result. However, the variation was high between subjects, especially on dry eye subjects. Comparing all the blink parameters, we found that the IBI was the most sensitive metric for differentiating the effect of concentration on a task and ocular surface stimulation on blinking, allowing us to use the IBI to study the blink response in Chapters 3 and 4.

The reason that the other blink parameters, including amplitude, velocity and duration, showed less clear cut effects on blinking under the conditions tested in this study were unclear. The number of subjects tested in this study was small and individual variation in all blink parameters was high.(Kaminer, Powers et al. 2011) In addition, dry eye subjects showed more individual variation in their blink parameters than did normal subjects. The purpose of Chapter 2 was to study a range of blink responses in dry eye to normal subjects, not to compare dry eye to normal subjects. However, the variability in blinking among the dry eye subjects included in this study was higher than we expected, which affected our ability to detect differences in blink parameters among the conditions tested. The reason for this high individual variation among dry eye subjects may be due to changes in the ocular surface with dry eye. Previous studies have found dry eye subjects had a desensitized surface,(Bourcier, Acosta et al. 2005) whereas other found hyperesthesia.(Situ, Simpson et al. 2008) It is possible that the tear film instability and

hyperosmolarity thought to be central to the dry eye condition may have damaged the ocular surface and altered underlying neural activities, leading to abnormal responses.(Belmonte, Acosta et al. 2004) This may have added to the variability or "noise" we found when studying the correlation between ocular surface inputs and blinking.

In Chapter 2, we found that the IBI, or the timing of each blink, was one of the most sensitive metrics to the different conditions we tested, along with blink duration. The reason why these blink parameters are the most responsive to various conditions is unclear, but may be related to the idea of the spontaneous blink generator, which is hypothetically located at the trigeminal complex in the brainstem. The spontaneous blink generator theoretically stimulates the blink after receiving direct afferents from ocular surface and indirect inputs from central nerve system (dopamine and cognitive concentration). However, the interplay between these inputs and the manner in which blink timing and other parameters are set remain unknown, requiring more studies in the future.

In Chapter 2, we theorized that ocular surface stimulation was involved in triggering blinking. However, this and previous studies in our lab have not found any significant correlation.(Himebaugh, Begley et al. 2009) This may be because normal blinking is not only triggered by corneal inputs, but also dopamine level that the increasing activation of dopamine system would increase the blink rate and decrease the blink amplitude, contributing to the overall poor correlation between ocular surface inputs and blink.(Goldberg, Maltz et al. 1987; Wichmann and DeLONG 2003; Kaminer,

Powers et al. 2011) In addition, we expected that tear break-up over the corneal surface should stimulate a blink due to surface irritation.(Himebaugh, Begley et al. 2009) However, we did not find an association, perhaps due to our method of analysis or possible ocular desensitization of some subjects included in this study (discussed above). Another reason may be that our measure of tear break-up and its increasing area may not be representative of the stimulus involved. It was possible that mechanical force from the air flow stimulus or overall tear film thinning may provide signals for blinks.(Begley, Simpson et al. 2013) The location of tear breakup may also be a factor.(Himebaugh, Begley et al. 2009) Himebaugh et al. found that the occurrence of tear break-up in the center of the cornea was more likely to trigger a blink, compared to that in the peripheral area. Thus, it is difficult to quantify the ocular surface signals that may be involved in blinking by simply imaging the tear film break-up. For that reason, we developed a method to measure the effect of increasing stimulation to the ocular surface in Chapter 3.

In Chapter 3, only healthy young subjects were recruited to avoid the effects of any damage to the ocular surface in dry eye, as well as potential irritation from rapid tear break-up in dry eye.(Bourcier, Acosta et al. 2005; Savini, Barboni et al. 2006; Situ, Simpson et al. 2008; Tuisku, Konttinen et al. 2008) Increasing levels of air flow with known rates were applied to the central cornea while subjects were engaged in a visual task (computer game). Thus, this study design sought to quantify the effects of stimulation to the ocular surface on blinking, while controlling the effects of attention on a task as much as possible.(Bourcier, Acosta et al. 2005; Cardona, Garcia et al. 2011)

One of the main purposes in Chapter 3 was to explore the correlation between ocular stimulation and blinking. Previous studies have shown that the IBI or BR was highly variable among healthy subjects when no stimulus was applied.(Doughty and Naase 2006) Most previous studies have found that IBI distribution is positively skewed with a few longer IBIs, but with increasing ocular surface stimulation tended to decrease the IBI.(Tsubota, Hata et al. 1996; Nakamori, Odawara et al. 1997; Acosta, Gallar et al. 1999) In this study, we found a linear relationship between external stimulus intensity and the average IBI duration, further suggesting the normal ocular surface could both detect external stimulation and modulate the blink frequency appropriately, presumably to protect the surface from potential damage.

The underling mechanism for the linear response of blinking might be due to the increasing ocular surface inputs generated by the neural receptors on the ocular surface. As mentioned in the introduction, all types of neural receptors are able to encode the magnitude of the stimulus by increasing their discharging rate. Thus, the increasing ocular surface signals increased the excitation in the trigeminal complex in the brainstem, which finally increased the blink rate. This result is also reasonable from the evolutionary point of view. Since we are living in a highly variable environment which changes all the time, it is necessary to develop a nerve system detecting external stimulation precisely and modulating the blink response properly to protect the ocular surface.

In Chapter 3, we found that ocular surface stimulation increased both the frequency and the regularity of blinking, so that the increased blinking with stimulation

occurred in short comparatively constant intervals.(Tsubota, Hata et al. 1996; Belmonte, Aracil et al. 2004) From the physiologic view, the more regular blinks might provide a more stable tear film for increased ocular surface protection. Previous studies have suggested that transient irritation of the ocular surface might increase trigeminal complex excitability, which is associated with blinking at relatively constant intervals, termed blink oscillations.(Peshori, Schicatano et al. 2001; Evinger, Bao et al. 2002; Kaminer, Powers et al. 2011) Although our experimental conditions differed in that we used continuous air stimulation, this may have produced multiple blink oscillations, resulting in an overall more regular blink pattern. The increasing regularity of blinking, in response to surface stimulation, may perhaps have acted as an adaptive modulation to provide both ocular surface protection and more rapid tear film renewal.(Peshori, Schicatano et al. 2001; Evinger, Bao et al. 2002)

In this study, we introduced a new term, the blink increase threshold (BIT), which was defined as the air stimulus intensity that triggered a statistically significant blink frequency change (measured by the IBI) from baseline. Considering the linear relationship between flow rate and IBI duration, it might seem unnecessary to induce this new term. We introduced it as a new endpoint that may have utility in measuring the abnormality of the ocular surface sensory response in dry eye. Because blinking is the normal protective response, decreased responses may be associated with dry eye, as we found in some subjects in Chapter 2. However, this new measure requires future testing in dry eye subjects to affirm its utility. Although the BIT is likely to depend on the integrity of the ocular surface sensory response, it probably differs from traditional corneal sensitivity measures, which are based on subjective sensation.(Belmonte, Acosta

et al. 1999) In this study, one subject did not report any ocular sensation from all the trials, however, his blink pattern was still changed significantly. Thus, the BIT might not induce a subjective sensation, but provides enough stimulation to the underlying receptors to modulate the blink. It also may be expected to vary with concentration levels, as many different previous studies have found the blink pattern was different when varying the difficulty of the task.(Cardona, Garcia et al. 2011) Thus, the cognitive state or task should be carefully controlled when measuring this threshold.

Ocular surface sensation also increased with increasing ocular surface stimulation, but the results were more variable than the blink response. Watery had the biggest increment in all the sensory ratings, probably due to tear secretion, which was expected (Stern, Gao et al. 2004; Situ and Simpson 2010), but not measured in this study. Cooling was probably induced by the room temperature air used in our stimulus, as well as likely increased evaporation in the tear film during stimulation. This temperature change might be detected by the cold thermoreceptors, modulating the tear secretion. Discomfort is considered a global sensation and may be due to a mixed input from all three types of receptors on the cornea.(Belmonte, Aracil et al. 2004) Although the initial afferent for blink and sensation is the same up the level of trigeminal complex in the brainstem, blink and sensation pathway then diverge.(Stapleton, Marfurt et al. 2013) Judgments of sensation involve higher centers in the brain, which are perhaps an additional basis for differing responses among individuals. However, despite these differences in sensory reports among subjects, the correlations between IBI and some ocular sensations were quite high for watery, discomfort and cooling sensations, underscoring the possibility of a common origin of blinking and the sensory response at the ocular surface.

In the Chapter 4, we used similar experimental setup as Chapter 3 to further study the dynamics of tear secretion, blinking and the timing of the responses, while applying different levels of ocular surface stimulation. A new metric, tear meniscus fluorescein concentration (TMFC), was developed to better study tear dynamics. Traditionally, the tear meniscus height (TMH) has been used to estimate tear secretion.(Mainstone, Bruce et al. 1996; Kawai, Yamada et al. 2007; Situ and Simpson 2010; Tung, Perin et al. 2014) However, TMH can be affected by blinking, in that a high blink rate will pump out the tears, masking the tear secretion effect on TMH. When this happens, the TMH may not appear to change even though new tears are rapidly being secreted. For that reason, we used fluorescein dye, which would show more rapid changes in intensity with increasing tear secretion, even if TMH did not change. Further, we calculated the TMFC from its intensity (Nichols, King-Smith et al. 2012) because fluorescein molecules can undergo quenching, so that any measured fluorescence intensity can represent concentrations above or below the critical concentration.(Webber and Jones 1986)

Although the TMFC gave more information about tear secretion when the TMH did not change due to increased blinking, it was also affected by blinking. In order to disengage blinking from tear secretion, we developed another novel metric, the IBI-TMFC slope, which measured the slope of the change in the calculated fluorescein dye concentration between each blink. This measure makes the assumption that changes in dye concentration between each blink are due to secretion, not tear drainage between blinks, which is thought to be minimal within 0.15sec after a blink.(Zhu and Chauhan 2005) Given the time measurements in this investigation, our study setup was very

unlikely to capture events in that time period. Thus, the IBI-TMFC slope provides an improved method for studying the tear secretion than either the TMH or TMFC metrics.

As we expected, both blinking and tear secretion were increased with surface stimulation, presumably to protect the eye from adverse situations.(Adler, Kaufman et al. 2011) However, the blink was increased linearly with air stimulation, whereas the tear secretion rate, measured by IBI-TMFC slope, was increased exponentially. One possible explanation was that different receptors might be involved in modulating the tear secretion, as the stimulus intensity increased. When the stimulus intensity was low, it is likely that only cold thermoreceptors were activated,(Murphy, Patel et al. 1996) and the tear secretion still remained close to its basal level.(Parra, Madrid et al. 2010) When the stimulus intensity was high, the polymodal and mechanical nociceptors may also have been activated, inducing reflex tearing(Belmonte, Aracil et al. 2004; Parra, Madrid et al. 2010) and significantly increasing the IBI-TMFC slope, contributing to the exponentially increasing pattern.

Comparisons between the blink and tearing responses were interesting. There was a time delay between these two responses, with blinking almost immediate and tearing several seconds later, on average. The reason for this time lag is unknown. Presumably, the initial pathway for signaling required similar timing for both responses, although it is possible that different neuron subclasses at the ocular surface were involved. (Stapleton, Marfurt et al. 2013) Likewise, transmission times to the muscles controlling the blink and glandular secretion of tears were likely to be relatively similar, at least compared to the rather large 5 second lag between the two responses noted in this study. Thus, the most plausible reasons for the delay in tear secretion are the time required for tear production

and/or the time required for the tears to appear in the lower meniscus following secretion. According to the literature, “aqueous tears are secreted into the supero-lateral fornix by the main and palpebral portion of the lacrimal gland and into the upper fornix by the accessory lacrimal glands.”(Gaffney, Tiffany et al. 2010) Thus, the delay in tearing might represent the time needed for tears to flow from the fornices to the meniscus following secretion.

Both responses showed adaptation to the stimulus, which probably related to the underling neural activities.(Holly, Lamberts et al. 1982; Holly, LauKaitis et al. 1984) Although the nature of the stimulation on the ocular surface was unknown, all the subclasses of receptors showed decreasing firing rate over time to the stimulus, which might result in the adaption found in this study. Sensory adaptation is widely found in other systems, such as tactile and auditory system. However, the physiologic reason is unclear. Theoretically, the stimulus intensity in this study was constant over time, thus both blink and tear secretion should remain high at the same time. Lowering both responses might potentially decrease the surface protection, initialing a sequence of changes for dry eye disease.

Although the underlying mechanism for these results are unclear, these data provide the basis for understanding the ocular surface auto-protective mechanisms in normal subjects, which could be used in the future to study changes associated with pathological conditions, such as dry eye.

In this thesis, we designed three studies to better understand the ocular surface blink, tearing and sensory responses to varying surface stimulation. As we expected,

these responses were highly correlated to the level of stimulation, since they all receive input from ocular surface. The sensory afferent fibers starting from the ocular surface project to the trigeminal complex in the brainstem though the trigeminal nerve. For here, the sensory fibers diverge, projecting to different regions for triggering blinking, tearing and pain sensation. The efferent fibers from the facial motor nucleus terminate at the orbicularis muscles to effect blinking. The efferent fibers from the superior salivatory nucleus, go through several inter-nucleuses and finally terminate at lacrimal gland to cause tearing. Some sensory fibers from the brainstem project to multiple locations in the higher centers of brain to create pain sensations.(Stapleton, Marfurt et al. 2013) Since the purpose of all these responses is to protect the ocular surface from adverse stimulation, all the responses should be increased with external simulation and correlated with each other.

In this thesis, we found blinking, tearing and ocular sensation were increased with surface stimulation, however, the correlation between these three was modest. The neural pathways for these three responses diverge at the trigeminal brainstem complex. The pathway for blink locates at low level, making blink as the fast response to stimulation. The ocular sensation involves high center nerve system modulations, which add in bias for the final sensation. Lacrimal gland is not only innervated by parasympathetic nerve fibers from superior salivary ganglion, which receive the signals from ocular surface, but also innervated by sympathetic nerve fibers originating from superior cervical ganglion. The different innervations might contribute to the modest correlation between them.

Although this thesis helps us to understand the protective responses under varying conditions, it also brings up many more questions, requiring further studies. For example, we when do not fully understand the stimulus used in our studies and the neural types stimulated by it. As mentioned in Chapter 3 and 4, a room temperature air flow can provide a mechanical pressure on the ocular surface, as well as chemical (hyperosmolarity) and thermal (cooling) stimulation due to evaporation. Therefore, the nature of any stimulus and its effects on the ocular surface are needed to improve our understanding of the abnormal blink and tear secretion responses found in dry eye or other conditions.

In addition, the manner in which different types of neurons contribute to the blink and tear secretion are still unclear. Recent studies have suggested the cold thermoreceptors played an important role in maintaining the basal tear secretion.(Parra, Madrid et al. 2010) However, the contributions of polymodal and mechanical nociceptors to reflex tearing, to our knowledge, have not been fully studied. In Chapter 4, we found the tear secretion was increased exponentially, whereas blinking increased linearly with the ocular surface stimulation used in our studies. Thus, the response of the ocular surface sensory neurons and the types activated with different stimuli will be helpful to explain this difference.

There was a five second delay between blinking and tearing responses. We speculated that the delay was due to the time required for the tears to appear in the lower meniscus following secretion. However, more studies are needed to understand the time

period from signals arriving at the lacrimal gland to new tears secreted, as well as the timing of the transportation pathway from the lacrimal gland to the ocular surface.

Another unexpected finding in this thesis was that both blinking and tear secretion showed adaptation over time, which, could be considered detrimental to ocular surface protection. Previous studies have shown decreasing neural firing rates over time with stimulation for all three types of corneal receptors, which might be the reason for the adaptation found in this study.(Gallar, Pozo et al. 1993; Chen, Gallar et al. 1995; Hirata and Meng 2010; Parra, Madrid et al. 2010) Although adaption is found in many other systems,(Westerman and Smith 1984) more studies are needed to understand the physiological reasons for this apparent adaptation. For example, perhaps the fullness of the blink increased with adaptation to the stimulus, or there may have been a velocity change, neither of which was measured in Chapter 4. This could act to spread a thicker tear film over the cornea to compensate for the lowering blink rate as the external stimulation continued. It is also possible that the lowered blink rate and tearing response with adaptation may be beneficial for quickly regaining good vision. Although we did not test vision in this study, previous studies have found that the best optical quality was not reached until several seconds after a blink when no reflex tearing has occurred.(Tapper 1965; Montes-Mico, Alio et al. 2004) Extra liquid flowing across the meniscus, such as in reflex tearing, is likely not to be a uniform thickness and thus probably acts to distort vision. Increased blinking may also interfere with completing a visual task, such as the one used in this study. Thus, the adaptive response we found for both blinking and tearing may serve to regain better vision quickly, which could be important from an evolutionary point of view.

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Ziwei Wu

School of Optometry, Indiana University Bloomington

ziwwu@indiana.edu

(812)-856-5915

Education

Ph. D in Vision Science *Sep. 2008 - Feb. 2015*

Indiana University Bloomington, IN, USA

B. S in Clinical Medicine (Optometry) *Sep. 2003 - Jul. 2008*

Tianjin Medical University, Tianjin, China

Ph.D Thesis Projects

The effects of ocular surface stimulation and task concentration on blink parameters *Sep. 2009 - Sep. 2010*

- Investigate the effects of concentrating on a visual task and a mild ocular surface stimulation on multiple blink parameters
- Quantify the effect of the ocular surface stimulus via tear film instability and determine whether the changes in tear film are associated with altered blinking patterns

The effects of increasing ocular surface stimulation on blinking and sensation *Jan. 2011 - Dec. 2011*

- Understand how increasing ocular surface stimulation affect blinking and

sensation

- Determine correlation between blinking and sensation

The effects of increasing ocular stimulation on the blinking and tear secretion *Jan. 2012 - Aug. 2014*

- Develop new metrics to quantify the tear dynamics over time
- Investigate the correlation between tear secretion rate and external surface stimulation
- Examine the interaction between blinking and tear secretion

Clinical Internship in China

Tianjin Eye Hospital *Apr. 2008 - Jul. 2008*

- Strabismus and Pediatric Ophthalmology center
- Optometry Clinic

Tianjin Medical University Eye Hospital *Jan. 2008 - Mar. 2008*

- Cataract center
- Refractive center
- Optometry Clinic

Hong Kong Polytechnic University *Oct. 2007 - Nov. 2007*

- Optometry Clinic

Lectures

- Wu Z. How do blinking and tear secretion respond to ocular surface stimulation? American Academy of Optometry, Denver. *Nov. 2014*
- Wu Z. The ocular surface controls over blinking, tearing and sensation. Indiana University Bloomington. *Sep. 2014*
- Wu Z. The effect of increasing ocular surface stimulation on blinking. Indiana University Bloomington. *Feb. 2013*
- Wu Z. Ocular surface controls of spontaneous blink during different concentration tasks. Indiana University Bloomington. *Feb. 2012*
- Wu Z. Controls over spontaneous blinking in dry eye and normal subjects. Indiana University Bloomington. *Mar. 2011*
- Wu Z. Blinking patterns and associated ocular surface under different conditions. Indiana University Bloomington. *Apr. 2010*

Publications

- Wu Z, Begley CG, Situ P, Simpson T. The effects of increasing ocular surface stimulation on blinking and sensation. Invest Opthalmol Vis Sci. 2014; 55; 1555-1563
- Wu Z, Begley CG, Situ P, Simpson T, Liu H. The Effects of Mild Ocular Surface Stimulation and Concentration on Spontaneous Blink Parameters. Current eye research. January 2014, Vol. 39, No. 1 , Pages 9-20

- Wu Z, Begley CG, Bradley A, Port N. The effects of increasing ocular surface stimulation on blinking and tear secretion. (paper submitted to Invest Ophthalmol Vis Sci)
- Begley C, Simpson T, Liu H, Salvo E, Wu Z, Bradley A, et al. Quantitative analysis of tear film fluorescence and discomfort during tear film instability and thinning. Invest Ophthalmol Vis Sci. 2013; 54(4):2645-53.

Published Abstracts

- Wu Z, Begley CG, Situ P, Simpson T. Blinking and the sensory response to increasing air stimulation at the ocular surface. ARVO 2014: Abstract# 3647
- Wu Z, Begley CG, Situ P, Winkeler AJ, Zhang J, Simpson T. How does increasing ocular surface stimulation affect blinking? ARVO 2013: Abstract# 4354.
- Wu Z, Begley CG, Zhang J, Simpson T. How does mild ocular surface stimulation affect spontaneous blinking? ARVO 2012: Abstract# 587.
- Wu Z, Begley C, Zhang J, Adebesi LA, Simpson T. Does ocular surface stimulation oppose internal controls over blinking? ARVO 2011: Abstract # 3846.
- Wu Z, Begley CG, Liu H, Zhang J, Adebisi L. Which blink parameters are sensitive to ocular surface stimulation and symptoms? AAO 2011: #115687.
- Wu A, Begley c, Liu H. Blinking patterns and associated ocular sensations under different conditions. ARVO 2010: Abstract# 2384.
- Wu Z, Begley CG, Himebaugh. Detection of edges of tear break-up imaged by different methods. ARVO 2009: Abstract #523.

- Zhang J, Begley CG, Thibos L, Simpson T, Wu Z. Visual disturbance and ocular irritation in an experimentally induced tear film instability model. ARVO 2014; Abstract #1989
- Situ P, Simpson T, Chalmers RL, Wu Z, Begley CG. Validation of the Current Symptom Questionnaire (CSQ): a meta-analytical approach. ARVO 2013 #6023.
- Begley CG, Zhang J, Situ P, Wu Z. The ocular surface sensory response to tear film instability with and without a contact lens. ARVO 2013; Abstract #905
- Zhang J, Wu Z, Simpson T, Begley CG. Evidence for fluorescence dye quenching of the tear film and its interpretation. ARVO 2012: Abstract# 4258/A135.
- Venezia L, Begley CG, Situ P, Wu Z, Cain E, Winkeler A, Zang J. Measuring tear turnover rates by monitoring fluorescein de-quenching. AAO 2012: #125674.
- Begley CG, Liu H, Wu Z, Simpson T, Salvo E. What does fluorescence quenching reveal about tear break-up versus slow thinning of the tear film? AAO 2011: #110688.
- Liu H, Begley CG, Simpson T, Zhang J, Jansen ME, Himebaugh N, Wu Z, Kollbaum P. Non-invasive high resolution imaging and objective quantification of contact lens wettability. ARVO 2011: Abstract # 6475/D939.
- Zhang J, Greenlee JD, Begley CG, Liu H, Simpson T, Chalmers RL, Wu Z, Himebaugh NL, Jansen ME. A novel contact lens wettability grading scale related changes in visual function. ARVO 2011: Abstract # 6529/D993.
- Liu H, Begley CG, Simpson T, Zhang J, Jansen M, Himebaugh NL, Wu Z. Non-invasive high resolution imaging and objective quantification of contact lens wettability. AAO 2011: #115661.

- Liu H, Begley CG, Himebaugh N, Thibos LN, Wu Z. Association of tear film wavefront metrics with grade of pre-lens tear break-up and vision. 6th Intern Conf on Tear Film & Oc Surf, Florence, Italy, Sept 2010.
- Kollbaum, PS, Begley CG, Springs C, Wu Z, Himebaugh NL. Simultaneous measurement of fluorescein tear film break-up wavefront optics, and visual function. ARVO 2009: Abstract #528.
- Himebaugh NL, Begley CG, Wu Z, Chalmers RL, Moody K. Application of RMS fit error for assessing pre-lens tear film break-up. ARVO 2009: Abstract #535.
- Begley CG, Salvo E, Wu Z, Himebaugh NL. Sensory response to tear instability. ARVO 2009: Abstract #4243.

Honors

2014 Best Student Scientific Program Presentation (AAO, Denver)	Nov. 2014
American Academy of Optometry Travel Grant	*Sep. 2014*
Indiana University Travel Grant for ARVO	*May. 2013*
American Academy of Optometry Travel Grant	*Oct. 2011*

Leadership

Vice President of Vision Science Graduate Student Organization *Sep. 2010- Sep. 2012*