Ocular Discomfort Upon Tear Drying

by

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Abstract

Purpose: Assess the relationship between tear film drying and sensation between blinks.

Methods: MATLAB sampled a slitlamp video camera, a potentiometer and a microphone while subjects kept one eye open for as long as possible. 23 subjects rated the intensity of the ocular sensation while video and voice data were collected simultaneously. The tear drying on the cornea was measured.

Results: The sensation was triphasic. Two linear functions described the latter 2 parts of the data ($r \geq 0.95$). The correlation between TBUT and the elbow in the time-discomfort function was 0.72. Extent of tear film drying was linearly correlated to time (median correlation = 0.88). The correlation between the discomfort elbow and image elbow was 0.93 with single data pair for each subject. Analysis of sensation characteristics showed significant differences between itching and burning for both intensity and time ($p = 0.03$ and $p = 0.02$ respectively).

Conclusions: Simultaneous recording of ocular surface appearance, discomfort intensity and attributes of sensation provide novel information about the development of discomfort during ocular surface drying. The rapid increase in discomfort proceeding blinking has been quantified and the relationship between the time course of drying and discomfort is elucidated.

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Dedication

I dedicate this work to the Varikooty family, especially to my wife and children for their love, patience and constant moral support through all these years.

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LITERATURE REVIEW:

1. Dry Eye and the Sensation of Ocular Dryness:

This thesis attempts to address the fundamental question, “Does the eye, directly sense Dryness?”

Dry eye disease is one of the most common complaints seen by ophthalmic specialists [1]. It is a condition that causes considerable morbidity and reduces the quality of the life an individual. Historically the term Dry Eye is attributed to Herik S.C. Sjogren. The NEI/ Industry workshop’s definition is that “Dry eye is a disorder of the tear film due to tear deficiency or excessive tear evaporation, which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort.” [2]. An alternative definition of dry eye has been proposed which states “Dry Eye is a disease of the ocular surface attributable to different disturbances of the natural function and protective mechanism of the external eye, leading to an unstable tear film during the open eye state” [3].

Both these definitions encompass the importance of the interaction between the ocular surface and the tear film. The tear film is now believed to be a hydrated mucous gel containing a wide range of proteins and growth factors to promote and develop the ocular surface [4]. These specialized functions of the “hydrated mucous gel” may probably have evolved over thousands of years. It is highly likely that in the early stages of the evolution of life, the mucous covering the surface of primitive aquatic life forms functioned to reduce the hydrodynamic drag due to friction, during the process of movements [5]. With the development of the phylogenetically ancient, innate immune system, specific nerves and their patterns evolved as the final result of selective responses to combinations of local cues and interactions with the surrounding nature [6, 7]. Any alterations in these natural adaptations may in certain situations, cause people to describe these medleys of resulting unpleasant sensations as the feeling of dryness. The sensation of dryness is perceived both in the cutaneous and the mucous surfaces of the body.

Cutaneous Dryness: What do we mean by the term Dry Skin?

The following discussion regarding cutaneous dryness attempts to understand the common complaint heard in clinical practice “my skin\mouth\eye feels dry”.

The most common dermatological problem amongst all ages is “dry rough and flaky skin” and yet the term “dry skin” has never been defined in a reproducible way [8-10]. The confusion is often between the concept of dry, meaning without water or moisture, and the feature of a rough and brittle scaly skin appearance. Seborrheic dermatitis, in which there is an abnormal increase in the amount of sebum secreted and discharged and often associated with greasy scales, is also considered a type of dry skin. The term rough skin and dry skin described the medical diagnosis of, a low sebum content better than Seborrheic dermatitis [11]. Studies have also shown that dry skin is aggravated during winter and in conditions of low humidity [12, 13]. It is frequently a sign of epidermal dysfunction and often due to predisposing factors such as lack of water in the stratum corneum, hyperproliferation of the epidermis, inadequate synthesis of the skin lipids and epithelial barrier damage, with all of these factors often having an influence on each other [14]. The functional changes in the dermis and the epidermis are responsible for the viscoelastic properties of the skin. In the skin the outer most layer of the stratum corneum consists of the horny layer of the corneocytes containing layers of keratin filled cells. The two important factors responsible for an intact barrier function and for the adherence of the horny layer of corneocytes in the epidermis of the skin which maintains adequate hydration are the morphomechanical force of the desmosomes formed by the cell membranes that interdigitate with those of neighboring cells and the functional force of the intercellular lipids that fill the space between the cells. The water content of the skin is also dependant on “natural moisturizing factors”, such as amino acids, lactic acid, pyrrolidone carboxylic acid and urocanic acid. These are mainly formed after the break down of filaggrin, which is a histidine rich interfilamentous matrix protein present in the corneocytes [15, 16]. An alteration in the filaggrin metabolism contributes to the disturbed epidermal differentiation leading to the pathological and adaptive changes of dry skin. In the eye, filaggrin is upregulated in the keratinized conjunctiva, but is not found in the normal conjunctiva [17]. The barrier function of the skin is also correlated with the presence of covalently bound ceramides in the skin.

These ceramides play an important role in the formation of the lamellar structures that are involved in the maintenance of the barrier function of the skin [18]. There are also differences in the amount of bound ceramide present between the stratum corneum of the skin, gingival stratum corneum and the intraoral stratum corneum in the pigs. Bound lipid is important for the formation of the multilamellar structures that are present between the corneocytes for the barrier function of the skin [19]. It has been established that the transepidermal water loss is greater when there is a reduction in the amount of bound ceramides in the skin.

There are at present numerous methods to quantify the drying and irritant tendency of treated or untreated skin. Structural alterations in the skin are being studied through various bioengineering methods based on electrophysiological principles. The most recent of the techniques for an in vivo assessment of the skin structure and state of hydration, involve nuclear magnetic resonance spectroscopy, transient thermal transfer and optical coherence tomography [20, 21].

Neural Mechanisms of Dry Skin Perception:

Dry skin is not well defined and does not always mean dry. Various aspects are inclusive in the expression of dryness. There are now attempts to distinguish the following opposing qualities of the skin and its sensation, such as dry skin vs. hydrated skin, rough skin vs. smooth skin and seborrheic skin vs. asteatotic skin [10]. It seems therefore that dry skin is a rough skin of multiple origins. To appreciate the perception of skin roughness, and to understand the various physical determinants of roughness, a multidimensional scaling to similarity judgements of different textured objects was done. Texture was believed to include the two strong dimensions of soft vs. hard and smooth vs. rough. It has been proposed that these dimensions can occur in different combinations. A third dimension, which includes sticky vs. slippery improves the fit of the multi-dimensional scaling in some individuals, pointing to the conclusion that the perception of texture has two strong dimensions and possibly one weak dimension [22]. In many of the experiments combining neurophysiological and psychophysical studies, the form and the texture components of the surface structure of a substance have been investigated. Form perception involves the perception of the geometric structure of a surface or object and its dimensionality (i.e. the

degrees of freedom) is large. Texture perception is of a low dimensionality and it correlates with the feel of a surface. Texture is thus dependant on the distributed statistical properties of a surface or material such as the increase in spacing of dots or ridges. Most studies have demonstrated that roughness perception depends on numerous factors such as shape, height, diameter, compliance, and density of the surface. The relationship of these factors is complex and non-linear [23]. It has also been observed that the velocity of scanning, the force of contact and the friction between the finger and a surface have a minor or no effect on roughness magnitude judgments. Although the neural mechanism of roughness has been widely investigated the neural basis for roughness perception in the skin is unclear.

Peripheral Afferents of Mechanoreception and their Psychophysical Channels:

Four cutaneous mechanoreceptive afferent types innervate the glabrous (nonhairy) skin. These afferents are characterized on the basis of their receptive field size, i.e. large vs. small and by the rate with which they adapt to a sustained indentation, i.e. rapid vs. slow. Adaptation and the receptive field size are the principle properties of these mechanoreceptive afferents innervating the glabrous skin. Adaptation refers to how the afferent fibers respond to a sustained skin indentation. In the case of the fast adapting (FA) nerves, the responses to a stimulus is generated when there is an active indentation of the skin but if the movement of the skin stops the action potentials are not generated, even if there is a sustained indentation and the skin is under a considerable force. In contrast the slowly adapting (SA) afferents respond while the skin is moving and also during the period of sustained indentation. With a steady indentation being maintained the discharge rate slowly decreases in frequency, over many seconds or minutes. The receptive field of a mechanoreceptor is the area of skin which when stimulated generates a response in the sensory neuron of that area. The boundary of the area will depend on the intensity of the stimulus used. The various afferents types of cutaneous nerves based on receptive field and adaptation are:

The afferents associated with the Merkel cell are the “slowly adapting type 1” (SA1) afferents with a small receptive field.

The afferents associated with the Ruffini receptors are the “slowly adapting type 2” (SA2) afferents with large receptive fields.

The rapidly adapting afferents associated with the Meissner mechanoreceptors are the “rapidly adapting afferents” (RA) with small receptive fields.

The rapidly adapting afferents associated with the Pacinian mechanoreceptors are the Pacinian afferents associated with large receptive fields [24-33].

The slowly adapting type1 (SA1) and the rapidly adapting (RA) afferent nerves in the skin, exist in relatively homogenous populations. Also based on a ‘four-channel’ model of mechanoreception, the nerve fibers are found to be peripheral physiological correlates of the perceptual process of the model of mechanoreception [34-38]. A channel is defined as an element that is tuned to a specific region of the energy spectrum of the stimulus to which the system responds. In a multichannel sensory system, different channels are tuned to different regions of the energy spectrum of the stimulus [39]. The four channel psychophysical model of tactile perception proposes that each channel consists of specific end organs, innervated by select groups of peripheral nerves and isolated activation of these nerves can produce a unitary sensation. The most sensitive channel signals the threshold stimuli and suprathreshold sensations are due a combination of the activity of the different channels. However all the four channels need not be activated for a suprathreshold sensory experience. The model does not require the existence of all the four channels, and only implies that the sense of touch utilizes information being transmitted by separate and independent channels. A unified perception of touch is due to the activity over the different channels being integrated.

The four psychophysical channels of tactile sensation are named as

1) Pacinian (P)
2) Non-Pacinian I (NP I)
3) Non-Pacinian II (NP II) and
4) Non-Pacinian III (NP III).

The physiological input to the P channel is the PC fibers. The sensory attribute, which is routinely attributed to the P channel, is “vibration” [38, 40] and the Pacinian corpuscle afferents are the inputs to this P channel [36, 41, 42]. The P channel, which operates in the frequency range of 40-800 Hz, has a maximum sensitivity range at about 300 Hz. The channel is sensitive to changes in the temperature of the skin surface and to changes in the size and duration of a stimulus [38, 39, 43-45]. The P channel is capable of both spatial and temporal summation, as changes in the size and duration of the stimulus produces a decrease in the threshold of the P channel. The non-Pacinian channel known as NP1 has “flutter” as its sensory attribute [40]. The input for this channel is the RA fibers which innervate the Meissner corpuscles [46]. The NP I channel is less sensitive to changes in stimulus frequency. This channel possesses sensitivity to a range of vibrating frequencies between 10 and 100 Hz [34, 37, 47] and is not affected by changes in temperature. It is also not affected by the changes in the size and duration of the stimulus and therefore does not display temporal or spatial summation [48]. The next non-Pacinian channel, NP II operates in the vibratory-frequency range similar to the P channel (15-400 Hz.), but at a lower sensitivity. This channel is temperature sensitive but lacks spatial summation. Although initially it was believed that the NP II had temporal summation, subsequent work has not proved the presence of a temporal summation [37, 49, 50]. The sensory attribute of the NP II is presently not known although stimulation of this channel causes a buzz like sensation in the frequency range of 100-500 Hz. The slowly adapting type II fibers are the physiological substrates sub-serving the channel with inputs from the Ruffini end organs. The last channel is the NP III channel which operates at low stimulus frequencies in the range of 0.4 and 100 Hz. The channel is affected by the skin surface temperature, but does not have the capabilities of spatial summation. The Merkel cell neurite complex and the slowly adapting type1 fibers mediate the NP III channel. The sensory attribute of this channel is “pressure” in the frequency range of 0.4 to 2.0 Hz [51]. Therefore the four-channel model of mechanoreception maintains that tactile experience is the result of the combined neural activity of one or more of the various mechanoreceptive channels. The particular combination is dependant on the stimulus. The crossing from one channel to another resulted in a break of the overall power function, and the functions obtained were scalloped in shape indicating a switching from one channel to another [52]. In the hairy skin the relationship

between mechanoreceptive afferents and tactile perception is still not clearly known. Mechanoreceptive C fiber afferents that are associated with unmyelinated axons have been described in the human nerves both on the face and the forearm [53-55]. These fibers have low threshold mechanoreceptor properties and are different from the high-threshold C afferents, commonly classified as nociceptors. They have a strong response to a slowly moving stimulus but a poor sensitivity to a fast moving stimulus. As the function of these low threshold C- fibers is not fully understood, it is speculated that their activity may be conditional on the activity of the myelinated afferents and related to adding a particular quality or tint to the sensation elicited by skin deformation [56]. It has been suggested that like the unmyelinated nociceptive fibers and thermoreceptive afferents, which function to contribute to a larger limbic system for the integrity of the self, the low threshold C afferents too may be closely related to the limbic system [56, 57].

Relation of Intensity of Stimulus to Magnitude of Sensation:

The intensity of a stimulus and the perceived magnitude of the tactile sensation have been studied for long, with pioneering work by Weber and Fechner. Studies concerning stimulus intensity to tactile sensitivity have been done through neurophysiological, psychophysical and microneurographic methods. These studies reveal that the sensory capacity of a human observer is determined by the functional properties of the sense organs of the skin, rather than by mechanisms in the central nervous system [31]. In relation to the power law of Stevens, the magnitude of the tactile sensations could be described by power functions. When the growth in the intensity of tactile sensations was made as a function of indentation depth and rate it was found that the most intense tactile sensation was perceived due to the fastest (10 mm/sec) rate of indentation and the slowest indentation produced the least sensation. For a slow rate of indentation, the exponent of the power function was different from the exponent for the fast rate of indentation. In the case of the fast rate of indentation, two functions with different slopes were required to describe the estimates of the growth in intensity of tactile sensations [58]. The intensity of the tactile stimulus is therefore determined by a combination of activity across the different fiber types and no simple relationship between the afferent response and the perceived magnitude of the sensation is present.

A Note about the evolution of the mucous surface to reduce the drag force:

As early life evolved, nektonic animals interacted with the water that surrounded them. To increase the chances of survival these animals developed adaptations to reduce the drag due to friction on the boundary layer close to the surface of the body. One of the drag reducing surface adaptations, was the animal's "surface excreting long-chain polymers" [5]. These polymers reduced the frictional drag through the mechanism of the Toms effect, wherein the addition of small amounts of polymers to a turbulent high flow in a pipe, reduces the pressure drop substantially below that of the fluid at the same flow rate [59]. Investigating the Toms effect due to long chain polymers in the mucous of two molluscs, the Red Sea sea hare *(Aplysia oculifera)* and the Pacific nut brown cowrie *(Cyprea spadicea)* it was found that the mucous of stationary molluscs which was elastic, changed to a highly viscous state when sliding movements were applied [60]. Two opinions have been put forth to explain the friction reducing effects of long chain polymers in mucous. The first is that polymers act as viscoelastic threads, steering water particles in the mainstream direction, to reduce turbulence. The second opinion is that pieces of polymer chains that are released mix with the fluid and reduces friction. As amniotes evolved, the lungs reduced the need for a cutaneous gas exchange and the mucous and the skin formed the anatomic barriers to provide a phylogenetically ancient, innate immune system [6]. Specific nerves and their patterns developed as the final result of selective responses to combinations of local cues and interactions with the surrounding nature.

2. Neural coding mechanisms of roughness in skin:

The afferent nerves may react through different neural codes for conveying information about roughness perception. In the skin, four types of neural coding mechanisms have been advanced as possible bases for the perception of roughness; these are intensive, modal, temporal, and spatial codes [61, 62]. The intensive neural code is a measure of the amount of the neural activity such as the mean impulse rate, and is independent of the neural activity distributed in time and in space. Although the perception of roughness is believed to exist on an intensive continuum, no consistent relationship has been found between the mean impulse rates and the roughness magnitude [63-65]. The modal neural code for roughness perception is based on the relative magnitude of the intensity of response between neuronal populations with different transducer properties [62]. There have been very few studies of the modal coding of texture and roughness and no simple linear combination of the rates across the different afferent classes of nerves, could account for the magnitude of roughness [63, 66]. However based on the linear relationship between firing rate in the peripheral afferent nerves and the roughness ratings, it could be determined that the slowly adapting type of afferent nerve fibers were the type, most likely to mediate roughness perception. The non-linear relationships between the afferent rates and the roughness ratings are yet to be completely understood and the extent to which different classes of the afferents stay separated in the central nervous system are even less understood [67]. The temporal and the spatial coding mechanisms for the perception of roughness are also not completely understood. Some investigators believe that perceived roughness associated with neural activation, is dependent on both a spatial variable and a temporal variable [68, 69]. Other researchers studying the combined psychophysical and neurophysiological coding mechanisms of roughness concluded that temporal variation was not correlated with the magnitude of roughness but spatial variation was closely correlated with the magnitude of roughness [70]. In a study of the spatial patterns of the neural activity evoked in peripheral fibers and cortical neurons in the areas 3b and area 1 of the primary somatosensory cortex of the alert rhesus monkey (Macaca mulatta) the responses of the slowly adapting neurons in the area 3b were spatially acute, suggesting that slowly adapting neurons played an important role in tactual pattern recognition, due to integration within the CNS rather than summation within

the individual afferent's receptive field [71, 72]. Various other studies also point to the conclusion that the neural code for the perception of roughness is a spatial variation in the slowly adapting type 1 (SA1) firing rates. The spatial variation was computed as the mean absolute difference in firing rates between SA1 afferents with receptive fields separated by ~1-2 mm[66] leading to the belief that the perception of roughness is a spatial variation in slowly adapting type 1 firing rates, with receptive field centers separated by ~2 mm [70]. Other investigators have hypothesized that there exist different physiological mechanisms for perceived coarse roughness being dependant on a spatial mechanism, and a vibratory mechanism for the fine surfaces and evidence has shown that a intense high frequency vibration, can make a relatively smooth surface feel less smooth [73, 74].

Perception of Moving Stimuli in the Skin:

It is not yet clearly understood, if an increasing rate of indentation produces increased magnitude estimation for the same indentation depth. One study reached this conclusion. The same has not been found in other studies where the findings point to the fact that the rate of indentation depth does not influence the magnitude estimate for both tangential forces and for normal forces [58, 75]. Studies pertaining to the stretching of the skin have shown that the SAI afferents and the SAII afferents both show a dynamic and static sensitivity to the stretch of the skin. Similarly the rapidly adapting type I afferents and the rapidly adapting type II afferents both respond to the initial phase of stretching of the skin [76].

It has been now shown that the shear forces applied in a normal direction to the skin can be assessed independently of the tangential force of application [77]. However in studies where the roughness magnitude in the finger of the hand was studied in relation to the kinesthetic output, the perception of the roughness magnitude for a given surface was comparable whether the finger moved over the surface or the surface moved over the stationary finger, and hence it is believed that perception of roughness is independent of the kinesthetic output.

While the above mentioned anatomical, psychometric and physiologic mechanisms of touch give an indication about the touch mechanisms, only recently have the actual molecules which convert mechanical stimuli into electrical signals been understood at the biochemical and the molecular levels.

Molecular mechanisms of mechanosensation and its relevance to the ocular surface:

The shape of the cells and its architecture is due to the presence of the bi-lipid cell membrane and the cytoskeleton. Any cytoprotective response to an applied force is due to the interaction of the plasma membrane and the cytoskeleton [78]. It has been proposed that natural cells are constructed according to the principles of tensegrity. Tensegrity is defined as stable three-dimensional structure consisting of members under tension that are contiguous and members under compression that are not [79]. The intracellular architecture of the cell is formed by the cytoskeleton composed of microfilaments, microtubules and intermediate filaments. The microfilaments of the cytoskeletal lattice inside the cell exert tension to pull the cell membrane and it internal constituents towards the nucleus. Opposing this inward pull are two forces one of which is outside the cell and the other force is inside the cell. The outside opposing force is exerted by the extra cellular matrix, the "focal adhesions" to the substrate and the basement membrane of the cell. The force opposing the compressive microfilaments inside the cell is the microtubules. The intermediate filaments form the interconnecting links between the microfilaments and the microtubules also distribute the tension conveyed to the cell [80]. The cell therefore mechanically stabilizes itself by balancing the opposing forces of tension and compression. Two phenomena, believed to exist in the living cells, are the phenomenon of prestress, and the phenomenon of stiffening. Prestress is a preexisting mechanical tension present within the living cell. Stiffening is a response phenomenon seen in tensegrity structures, where the components of the structure reorient themselves in the direction of the applied stress and exhibit cell hardening. A strong association between prestress and the stiffness response of a cell has been recently demonstrated [80, 81]. The mechanosensitive ion channels (MSCs) were first described in skeletal muscle but are now identified in almost every type of cell in living organisms [82]. There are two major types of mechanosensitive ion channels. These are the stretch-activated channels (SACs) found commonly and the less common stretch-inactivated ion channels (SICs) which have been found only in the neurons and in the smooth muscle [83, 84]. The activation of a mechanosensitive channel requires that the channel's energy exceed the barrier(s) separating open from closed state. Any force, which is applied, does not directly *drag open* the mechanosensitive (MSC) channels but a mechanical stimulus

alters the probability of the channel to being open due to a change in the energy levels of the channel on account of the applied force. The stresses applied to the tissue could be in any direction and not just in the plane of the membrane as the channels themselves exert forces that are normal to the plane of the membrane [85]. The following hypotheses have been proposed to explain how movement activates mechanoreceptors.

The bilayer tension directly activates the channels. This is seen in the case of the stretch activated mechanosensitive (MScl) channel.

Mechanosensation could be due to the liberation of an extracellular ligand, which activates the mechanosensitive channel. In the case of some mechanosensory nerve endings such as the Merkel cells and the Pacinian corpuscles synaptic vesicles beside the nerve endings are present and it is believed that these synaptic vesicles might have a role in the formation of the extracellular ligands. Further proof of this is that destruction of the Merkel cell but not the adjacent and related nerve has failed to abolish mechanosensation leading to the suggestion that the Merkel cell is not a mechanosensory transducer [28, 86-88].

The third mechanism of mechanoreceptor activation is through the DEG/ENaC channel which binds the extracellular matrix and the intracellular cytoskeleton [89]. DEG/ENaC proteins belong to the degenerin/epithelial Na^+ channels. These proteins reside in many tissues including nerve endings. The proteins all share a common protein structure but differ in ion selectivity. Recently the brain sodium channel 1, which belongs to the family of degenerins, has been identified in the dorsal root ganglion and the protein has been traced in the mechanoreceptors located in the cutaneous tissue, including the Merkel and the Meissner corpuscles [90]. The movement of this complex DEG/ENaC channel amplifies and transmits the stresses that are applied, and it gates the mechanosensitive channels. In lower animals these DEG/ENaC channels respond to a mechanical stimulus by interacting with proteins in the surrounding matrix. In the mammals similar interacting extracellular matrix proteins are yet to be identified.

A mechanical stimulus to the cell causes a chain of biochemical events to occur in the cell. These sequences of events occur in a few seconds to a few minutes. An influx of $Ca2^+$ through the mechanosensory channels seems to be one of the first steps in mechanosensation

[91]. Events include, G protein activation, protein phosphorylation, secretion of growth factors, alterations in the cytoskeleton (CSK), changes in the cell-extracellular matrix adhesions, changes in the gene expression and importantly the release of chemical second messengers such as arachidonic acid, cyclic AMP, inositol triphosphate, and calcium [92]. In the human chondrocytes and in the periodontal ligament, the cells respond to mechanical stress by an increased production of prostaglandin E. The production of prostaglandin E is enhanced by cytokine interleukin-1 beta [93]. In conditions of retinal detachment, and in the case of the retinal pigment epithelium (RPE) cells and fibroblasts in tissue culture, application of mechanical stress causes the release of proteases [94]. In the bovine epithelial lens cell, the application of mechanical stress resulted in an increased permeability of intracellular Ca2+. The fibroblasts in the scleral and the corneal tissue may respond in a similar manner by causing the release of protineases [94].

Intercellular adhesions and gap junctions are another route through which a stimulus may be conveyed to the other cells which are part of the intercellular complex. Cell-cell adhesion molecules, such as cadherin mediated intercellular adhesion complexes connect cells to each other. Gap junctions, present between the cells, are intercellular transmembrane channels, which function to chemically connect the cytoplasm of neighboring cells. In response to mechanical stimulation of a single cell, the intercellular free calcium waves in the cells can travel over 10-20 cells through the gap junctions, from the site of origin of the stimulus. This is achieved by an increase in the level of phospholipase C. The phospholipase A and phospholipase C enzymes are termed as mechanosensitive enzymes and these enzymes through a sequence of biochemical events raise the level of inositol phosphate causing the release of calcium from intracellular stores for the propagation of the calcium wave [95]. Other molecular mechanisms proposed for the passage of a mechanical impulse are the changes caused by integrins. Integrins are a family of the cell surface proteins, which mediate cell adhesion and take part in cell-to-cell, and cell-to-matrix communication. Integrins can increase the inositol phosphate production by controlling the synthesis of the inositol lipid substrate, phosphatidylinositol bis-phosphate (PIP2) [96, 97]. Integrins also cause multiple signaling molecules to be associated with the cytoskeletal framework and the focal adhesion complexes. Hence mechanical stresses on the cells may generate chemical signals through more than one mechanism. Recent studies also indicate that intercellular

junctional proteins play a role in transducing physical forces into regulatory signals. Through the process of magnetic twisting cytometry, mechanical stresses were applied directly to the cell surface integrin receptors that were a part of the focal adhesion protein complexes. This resulted in a rapid and specific movement and localization of mRNAs and ribosomes towards the integrins. Following the localization of mRNAs there is a translation of mRNAs into proteins, near the site of reception of a signal. Mechanical tension and restructuring of the intracellular lattice may guide this movement [98]. The intracellular cytoskeleton also interconnects the extracellular matrix and the neighboring cells through the focal adhesion complexes at the cell base and the specialized junctional complexes at the lateral cell borders. As a result there is a molecular continuum and molecules in the extracellular matrix, the cytoplasm and the nucleus may be all mechanically coupled [92]. On the application of a highly localized force, it has been observed that the cells undergo deformation and the protruding margins of the deformed cells are mechanically coupled to the neighboring cells by the adherens junctions. Adherens junctions thus may directly transmit mechanical forces to the adjacent cells [99]. The application of a mechanical force to the adherens junctions activates the stretch sensitive calcium permeable channels and increases the intercellular actin polymerization [100]. In the eye, following a mechanical stress there is an increase in the levels of Transforming growth factor (TGF)-beta2, integrin beta1 and tenascin (TN) [101].

Thus there are various signaling pathways following a mechanical stress and these pathways interact with each other to produce a complex response to mechanical and other stresses.

3. Epidemiology of Dry Eye, Dry Skin and Dry Mouth:

Epidemiology of Dry Skin:

There is no universally accepted definition of the term "Dry skin" although one definition states "Dry skin is not a unique, well-defined condition but represents a medley of totally unrelated changes in the structure of the stratum corneum associated" [10]. Some of the common features seen in the condition of dry skin are:

1) Sensory characteristics with dry, uncomfortable, painful, itchy, stinging, and tingling sensation.
2) Tactile characteristics with a rough, uneven, and sand like feeling and
3) Visible characteristics with redness; lackluster surface; dry, white patches; flaky appearance; cracks; and even fissures.

The condition of dry skin is basically a sign of dysfunction of the epidermis and especially the stratum corneum. The meaning of the term dryness is different when mentioned in a subjective self-assessment by patients and during clinical assessment by a physician, dermatologist or an eye care professional. Dryness of the skin often is due to diminished water content of the stratum corneum of the skin caused by various factors (e.g. decreased perspiration, wind, low humidity, atopic dermatitis, vitamin A deficiency, etc). In a study consisting of 72 healthy volunteers, 67% had subjective complaints of dry skin, while only 5.6% had definite clinical signs of dry skin at the time of examination. Subjective complaints were more common in women than in men ($p < 0.001$), though neither clinical nor objective measurements showed any sex difference, indicating that multiple factors were responsible for the reporting of symptoms of dryness in men and women [102]. The subjective sensation of dryness was also rated in a self-assessment questionnaire sent to 3300 women and 500 men who were above the age of 18 and randomly selected, and 26.43% of females described themselves as having dry skin [103]. It was also established that symptoms of dry skin in association with dry throat and dry eyes were consistently related to dust particles and noise symptoms in office environments [104, 105]. Perceived sensation of skin dryness was also correlated with dermatological tests, in 925 persons, using semi quantitative dermatological methods [11]. A significant association was found between

the self-reported symptoms of dry skin and medical tests done to evaluate the symptoms. In terms of the skin symptoms, although the association between rough skin and the state of the skin's hydration is complex and remains to be settled, it was found that a low sebaceous secretion and/or a low stratum corneum hydration was significantly associated with skin complaints. The items dry skin and rough skin on the sensory perception subscales correlated well with the diagnosis of low sebum content [11]. In another selected population of 163 video display terminal workers, it was found that psychosocial factors were associated with an increased risk of reporting skin symptoms [106]. Studies with the scanning electron microscope have also revealed that in the dry atopic skin, the parameters of skin roughness were significantly increased, and in children there was a linear relationship of skin roughness to skin dryness ($p=0.02$) [107, 108]. The skin dryness is graded into four categories ranging from 0 to 3 points. Absent (0) = no symptoms of dry skin; mild (1) = ashiness, but no discernible flakes; moderate(2) = small to medium flakes; severe (3) = large flakes and prominent "cracked glass pattern" [109]. The population with skin dryness with grade 0, 1, 2 and 3 was associated with eczema in 22.3%, 39.9%, 57.0%, and 66.7% respectively, in a study population of children. The skin surface pH is normally in the range of 5.4-5.9, as measured on the skin of the volar forearm [110]. In conditions causing skin dryness there is often an increase in the pH value of the skin. In children the surface pH is lower than that in adults, with a study group reporting a mean pH value of 5.18. The pH values of uninvolved skin in the children with eczema showed a shift towards alkalinity, compared to children without eczema. Socio-demographic factors such as marital status, education, profession, income level and social class also have an influence on the experience of dryness symptoms [111]. The causes of skin dryness are thus multiple and many of the causes are to be discovered.

Oral Dryness – What do we mean by the term Dry Mouth?

The subjective complaint of dry mouth is termed "xerostomia". Xerostomia or oral dryness may be either due to salivary or non-salivary causes. Salivary causes may be due to diminished saliva, or due to an altered salivary composition. In a study of about 100 consecutive patients referred to a xerostomia clinic, idiopathic causes of xerostomia were noted in 21 patients while medical causes could be attributed to the others, but in the elderly

age group complaining of xerostomia most cases were associated with either medical conditions or drug therapy [112-114]. The subjective complaints of oral dryness are also related to palatal salivary secretion (PAL, measured as, microL/cm2/min) and parotid salivary flow (PAR, measured as, ml/min). It is demonstrated that most patients with a PAL resting flow rate of < or = 6.0 microliters/cm2 suffered from dry mouth, burning mouth syndrome or oral dysaesthesia [115]. Although an impaired salivary gland function is often associated with an impaired salivary gland secretion, there is no firm correlation between the two indicating that both the quantitative and qualitative properties of saliva play an important role in the perception of oral dryness [116].

The presence of mechanoreceptors in the different parts of the oral cavity has been documented. By employing the cytokeratin polypeptide marker CK 20 which is specific to the Merkel cells and the taste buds in the mouth, Merkel cells have been demonstrated in the oral cavity in the regions of, the mandibular gingival mucosa, the hard palate, the buccal mucosa and the lateral border of the tongue [117]. Studying the nerve fascicles of the human lingual nerve using microneurography it is shown that the superficial units of the lingual nerve, which terminate near the surface of the tongue, are of three different classes. These are the rapidly adapting nerve bundles, which resemble the fast adapting type 1 (FA1) nerve fibers of the glabrous skin of the hand and the slowly adapting units which, resemble the slowly adapting type I (SAI) and the slowly adapting II (SAII) afferents of the hand. The deep units encode information pertaining to the position of the tongue [117].

In spite of the demonstration of mechanoreceptor nerves and Merkel endings in the oral mucosa, the perception of drying and puckering in the oral cavity is not completely understood. These oral sensations of drying and puckering are referred to as astringency. Astringency is defined as “the complex of sensations due to shrinking drawing or puckering of epithelium as a result of exposure to substances such as alums or tannins” [118]. Astringency is also related to the ability of some chemicals to precipitate or cross-link salivary glycoproteins and epithelial bound proteins [115]. It is therefore perceived as “a resistance to movement in the form of roughness and dryness, and a feeling that the surface tissues of the tongue, palate, and lips are constricted or drawn” [119].

Psychophysical experiments have now established that astringency is a tactile sensation that results from the stimulation of mechanoreceptors during movement of the oral mucosa. The

stimulation of the mechanoreceptors is intensified both in the temporal and spatial domain when astringent compounds precipitate salivary mucin matrix. The cross-linking of the salivary mucin matrix possibly increases tension within the epithelium to stimulate the mechanoreceptors without need for contact between membranes. Another mechanism of tactile perception suggested is that alterations of the surface proteins could change the surface properties of the mucosa leading to an increased friction perceived texture [119, 120]. The human saliva also contains three major classes of proline rich proteins which have a high nonspecific binding affinity to tannins, which are plant derived polyphenolic compounds present in plant products and beverages. These complexes formed by binding are highly stable and the tannin-proline rich protein complexes cause a loss of lubrication and result in astringency both by decreasing viscosity and increasing friction [121-124].

Therefore the perception of oral dryness is a complex symptom to evaluate but the sensation of astringency may be due to the precipitation of proteins, although how the precipitation causes dryness is not fully understood.

Ocular Drying: What do we mean by the term Dry eye?

The Epidemiology of Dry Mouth and Dry Eye (ICD-9 #375.15):

Subjects complaining of ocular discomfort and dryness often have associated symptoms of skin and mucosal dryness. Such symptoms are especially prominent in conditions such as the sicca syndrome. In view of the multiple tissue involvement in circumstances leading to subjective symptoms of dryness, there is often a need for multidisciplinary offices to evaluate complaints of dryness. Dry mouth and dry eye are complaints, which often have an impact on the individual's quality of life. As there are no uniform parameters to evaluate dry mouth and dry eye, a comparison of the different values are difficult. There is often a higher prevalence of the symptoms of dry eye and ocular irritation in people confined to closed environments and numerous studies have found a strong association between the "indoor air quality" factors and perceived symptoms of Sick Building Syndrome. A study of commercial buildings in the US pointed out a strong association between skin problems, mucosal irritation, indoor air quality in buildings, and different psychosocial factors. The study reported that the percentage of participants reporting 'Dry Itching or Irritated Eyes' ranged from 24% to 52% with the overall percentage being 41%. The prevalence of other

mucosal symptoms of dry throat and stuffy nose ranged from 22% to 25% [105]. In an unselected clinical population of 1,054 patients in USA and Canada, dry eye questionnaires were used to evaluate ocular symptoms, and the most common ocular symptom was discomfort, with 64% of the non-contact lens wearers and 79% of the contact lens wearers reporting the symptom at least infrequently. 22% percent of non-contact lens wearers and 15% of contact lens wearers were diagnosed with dry eye. There was also a diurnal increase in the intensity of symptoms such as discomfort and dryness [125]. A population based survey of 2,520 subjects in the older age group reported that approximately 27% of the subjects reported dry eye or dry mouth to be present at all times, and 4.4% of the population reported both dry eye and dry mouth. About 14.6% of the subjects reported symptoms of dry eye. In a related study done separately on the same 2,520 subjects17% of the subjects reported symptoms of dryness of the mouth, with more severe symptoms and more frequent symptoms being reported in women. Dry eye was assessed on the basis of a questionnaire which asked about six symptoms; dryness, grittiness/sandiness, burning, redness, crusting on the lashes and eye being stuck in the morning. A person was considered to have Dry Eye Syndrome if the frequency of one of the six symptoms was reported as 'often' [126-128]. The Beaver Dam Study was a cross-sectional study done in the U.S. in 3722 men and women. Dry eye was defined as a positive response to a single question. An overall prevalence of dry eye was reported in 14.4% of the population between 48 to 91 years of age. The age adjusted prevalence was 11.4% in men and 16.7% in the women [129]. In a study of patients reporting to optometric practices in Canada, from a total of 3,716 patients 28.7% reported symptoms of dry eye, of these who reported dry eye, 24.2% reported concurrent dry mouth and 24.5% had symptoms, which were worse in the morning. The prevalence of patients reporting any level of symptoms of dry eye was 1 in 4 and severe symptoms were reported by 1 in 225 patients. The CANDEES study further reported sex related prevalence and amongst those with severe dry eye were mainly females in a ratio of 4.6 to 1 [130, 131]. Cross-sectional prevalence studies in Australia on 926 people reported that dry eye established through clinical tests was present in 10%-16.4% of the subjects. Severe symptoms of dry eye were reported more by women [132]. In a study done on 1,246 commercial pilots, 901 (72.6%) reported symptoms of dry eye during the flights, while only 67 (5.4%) reported symptoms of dry eye independent of the flight [133]. In Norway, the

epidemiology of dry mouth and dry eyes were studied by examining patients who listed in the rheumatoid arthritis register. 636 subjects between the ages of 20-70 years were examined. Symptoms of dry eye were reported in 38% of the subjects, oral sicca symptoms were seen in 50% of the subjects examined and both symptoms were noted in 27% of the subjects. More symptoms were noted in the groups who had reduced tears, reduced saliva or both, compared with subjects who had a normal tear and saliva secretion [134]. A symptom survey questionnaire combined with clinical tests was evaluated in a sample of 504 persons aged 30 to 60 in Copenhagen. Symptoms of dry eye were present in 24% of the population while symptoms of dry mouth were seen in 25% of the population. In this study complaints of oral and ocular dryness was particularly more common in women [135]. In Sweden, a study conducted on 705 subjects between the ages of 52 to 72 years reported symptoms of dry eye and/or dry mouth in 35% of the population and the prevalence rate was calculated to be 14.9% for keratoconjunctivitis sicca and 5.5% for xerostomia [136, 137]. Responding to a symptom survey, from a population of 2,500 people who were randomly selected from the general population in Japan, as many as 33% of the participants complained of Dry Eye while another study in Japan evaluated 2,127 new outpatients who reported to the clinic and symptoms of Dry Eye were noted in 17% of the sample population [138, 139]. Also, in studies across 14 office buildings in Germany, 817 patients were examined and the prevalence of symptoms of inflamed eyes, red eyes, irritated eyes and Dry Eye were 22.1%, 21.1%, 18.6% and 17.8% respectively [11]. Systemic autoimmune diseases such as Rheumatoid arthritis, juvenile arthritis, Sjogren’s syndrome, the spondyloarthropathies, systemic lupus erythematosus, multiple sclerosis, giant cell arteritis, and Graves' disease often have an ocular involvement. In Thailand a study of 224 patients suffering from rheumatic diseases, reported that 19.9% of the patients had dry eye [140]. The tables 3-1 to 3-4 indicate the prevalence of dry eye in different countries. The epidemiological studies reveal that the syndrome of Dry Eye is a common condition and the prevalence of this condition increases with age and is greater in the female population although in one study the prevalence of Dry Eye was more in the males. The data do indicate the increasing prevalence of Dry Eye although in many cases the etiological factors responsible for Dry Eye are unknown.

Factors Associated with Dry Eye:

The symptoms of Dry Eye are often exacerbated by the use of systemic medications such as the hydrochlorthiazides (diuretics), beta receptor blocking agents, the tricyclic antidepressants, antianxiety agents (psychotropic agents), and the antispasmodic anticholinergic agents [141, 142]. Environmental conditions responsible include reduced humidity, evaporative loss from wind, room heating or air conditioning, poor indoor air quality as seen in the sick building syndrome. A few of the systemic conditions associated with Dry Eye include Sjogren's syndrome, rosacea, systemic lupus erythematosus, rheumatoid arthritis, juvenile rheumatoid arthritis, spondyloarthropathies, multiple sclerosis, gaint cell arteritis, lymphomas, sarcoma and infection due to the human immunodeficiency virus. Conditions such as cicatricial pemphigoid and Stevens-Johnson syndrome produce a tear deficiency, due to glandular destruction of the goblet cells of the conjunctiva [140, 143].

The available data suggest that the syndrome of Dry Eye is commonly present. In spite of the many studies the data available are not a reflection of the true prevalence of ocular discomfort. The available data from less than 20 countries do not contain any information pertaining to the possible prevalence of Dry Eye in the densely populated countries in Asia such as China, India and Indonesia. At present there are no data available regarding the prevalence of Dry Eye in these countries. Also the lack of standardization in the terminology and diagnostic tests for evaluation of Dry Eye does not permit a point by point comparison of the different epidemiological studies. The larger epidemiologic picture clearly indicates the wide prevalence of Dry Eye in the community. From the point of view of health policy planning the innumerable symptom surveys and studies on Dry Eye and ocular discomfort indicate that the prevalence of this condition that is often regarded as trivial complaint will increase in the future. The table below summarizes many of the symptom surveys and studies reported about Dry Eye and ocular discomfort.

Epidemiology of Dry Eye and Ocular Irritation:

Serial No.	Country\Year Investigator	Population Size	Prevalence Rates of Dryness			
			M (%)	F (%)	Oral (%)	Eye (Dry Eye, discomfort) (%) and nature of study
1	Australia, 1998 McCarty et al. [132]	926	46.8	53.2		10.8% -16.3%-Dry Eye
2	Australia, 2000 McCarty et al. [133]	1246	1223	23		72.3% Pilots in flight-Dry Eye 5.4% independent of flight-Dry Eye
3	Australia, 2000 Albietz, [144]	1584				10.8% - Overall Prevalence 7.3% in < 40 yrs 18.1% in > 40 yrs
4	Canada, 1997 CANDEES Doughty et al. [130, 131]	13517	39.3	60.7		28.7% - Overall Prevalence 21.7% in those without contact lens, 50.1% in those with contact lens. 4.6:1 - Female to Male ratio
5	Canada, 2000 Begley et al. [145]	83				Ocular discomfort- found to be most common symptom in contact lens wearers
6	Denmark, 1997 Copenhagen City Heart Study Bjerrum et al. [135]	504			25	11% of the sample 24% reported eye symptoms
7	Denmark, 1998 Norn [146]					Dry eye was recorded in original patient records during the year 1930
8	Germany, 1977 Ruprecht et. [147, 148]	5,833	62.3	37.7		11.7% -Overall prevalence 15.1% in women 9.7% in men

Table 3-1: Epidemiology of Dry Eye

Serial No.	Country\Year Investigator	Population Size	Prevalence Rates of Dryness			
			M (%)	F (%)	Oral (%)	Eye (Dry Eye, discomfort) (%) and nature of study
9	Greece, 1997 Dafni et al. [149]	837		100		0.6% with dry eye and dry mouth in a closed rural community
10	Hungary, 1992 Hollo [150]	18				15 out of 18 patients had severe Dry eye along with Primary biliary cirrhosis
11	Indonesia, 2002 Lee et al. [151]	1058	52.3	47.7		27.5% - Overall Prevalence 22.8% in females 32.7% in males
12	Italy, 2000 Fenga et al.[152, 153]	213				72.3% -Lack of eye comfort Environmental survey of surgical operating rooms
13	Italy, 2001, Versura et al. [154]	1200				57.1% - Overall prevalence
14	Japan, 1993 , Toda et al. [155]	524	37.3	62.7		15.3% had dry eye
16	Japan, 1995, Hikichi [139]	2127				17% - Overall prevalence
17	Japan, 1999 Shimmura et al. [138]	2500				33% - Overall prevalence
18	Malaysia, 2000 Soo et al. [156]	52	2	98		(Study of patients with inactive SLE) 31% - Overall prevalence of dry eye
19	Norway, 1999 Uhlig et. al. [134]	636			50%	38% - Dry Eye 27% - Both dry eye and dry mouth

Table 3-2: Epidemiology of Dry Eye….continued

Serial No.	Country\Year Investigator	Population Size	Prevalence Rates of Dryness			
			M (%)	F (%)	Oral (%)	Eye (Dry Eye, discomfort) (%) and nature of study
20	South Africa, 1995 Bulbulia et al. [157]	78*	94	6		40% - Overall prevalence of dry eye in chemical workers.
21	Sweden, 1989, Jacobsson et al [136]	705			5.5	14.9% reported KCS, 35% reported either Dry eye or Dry mouth
22	Thailand, 2002 Ausayakhun et al [140]	224				19.9% -Overall prevalence of Dry eye
23	United States, SEE Project 1997-1999 Bandeen-Roche, Schein et al., Hochberg et al.[126, 127, 158]	2520	42*	58*	17*	14.6% of sample (65-69 yrs) 16% of sample (>80 yrs) 27% reported either dry eye or dry mouth at all times. 4.4% had both.
24	United States, 2000 Moss et al. [129]	3722	43	57		14.4% - Overall prevalence 11.4% in Men 16.7% in Women
25	United States, 2000-2001 Schaumberg et al, WHS* [159, 160]	39,876 (WHS)		100		6.7% - Overall prevalence (for WHS) 5.9% - 9.1% (users of HRT)
26	United States, 2000-2001 Schaumberg et al, PHS** [159, 160]	22,071 (PHS)	100			2.3% clinically diagnosed DES 1.9% -severe symptoms
* Women's Health Study ** Physician's Health Study						

Table 3-3: Epidemiology of Dry Eye….continued

Serial No.	Country\Year Investigator	Population Size	Prevalence Rates of Dryness			
			M (%)	F (%)	Oral (%)	Eye (Dry Eye, discomfort) (%) and nature of study
27	United States, 2001 Yazdani et. al. (from records) [161]	25,180 (1997) 27, 289 (1998)				0.4% - 0.5% is Overall prevalence of treated eye disease.
28	America, 2001 Reynolds et al. [105]	697			22 (13% had dry skin associated with the symptoms of dry eye)	41% had dry, itching or irritated eye 42% had tired or strained eye
29	North America (US and Canada), 2001 Begley et al. [125]	1054	36	64		22% Non-contact lens users-had Dry Eye 15% Contact Lens users-had Dry Eye

Table 3-4: Epidemiology of Dry Eye….continued

4. Measurement of Ocular Discomfort:

The history of pain and nociception is perhaps as old as mankind. Although it is a major cause of morbidity in the world only recently has its importance been realized in a partial way by health care professionals and the public. The World Health Organization has established guidelines for cancer pain relief. However to address the issues of discomfort and pain due to the various causes in different parts of the world, there is no unified approach. It is therefore not too surprising that ocular discomfort in spite of its world-wide prevalence has not received adequate attention. During the past 20 years, interdisciplinary scientific organizations have been formed to advance the understanding of the ocular surface and also for the management of pain. Two important phases in the study of pain were:

1) The realization that expression of pain was not just due to tissue damage or disease but due to the interaction of various factors such as prior learning history, cultural background, and environmental and social conditions.
2) Randomized controlled studies replacing the uncontrolled studies that were previously being done [162].

A need for accurate measurement of pain emerged from concepts which permitted the measurement of attributes which seemed immeasurable and the assignment of numbers to events which led to the development of measurement scales [163]. A four-fold classification of scales was first presented at the International Congress for the Unity of Science in 1941. Its publication (delayed due to World War II) distinguishes four main classes of scales. A fifth scale termed as the Logarithmic interval scale was proposed later.

The four classes of scales are:

1) A nominal scale identifying names and having no metric information with its empirical operation being the determination of equality. Only a few statistics are valid on a nominal scale measurement such as category frequencies, mode and sometimes contingency coefficients.

2) An ordinal scale to rank items with no information about distances between values with its empirical operation being determination of greater or less. Statistics possible on an ordinal scale are median, centiles, and rank order statistics.

3) An interval scale with equal units but lacking a meaningful zero point with the empirical operation being determination of the equality of intervals. The coefficient of variation is not valid on an interval scale while all other statistics are valid.

4) A ratio scale having defined intervals and with a true zero point with its empirical operation being, the determination of the equality of ratios [163-165].

Between the periods of 1929 to the early 1950s a search for effective non-narcotic analgesics led to development of a scale of pain measurement based on the "jnd" or just noticeable difference. This scale termed the "dol scale" quantified the pain threshold as a function of the stimulus intensity where one "dol" was the equivalent of two jnds [166]. The dol scale was challenged with the realization that emotion played an important part in response to pain and this led to the development of other scales such as an ordinal scale and a paired comparison method to assess pain [167]. Elsewhere other scaling techniques were being developed and the stimuli were divided into two types. Those forming prothetic continua were concerned with the 'how much' or the quantitative aspects, and the metathetic continua were concerned with 'what kind' or the qualitative aspect of a stimulus. Three types of scales were developed and the differences between the prothetic and metathetic continua were demonstrated. The three types of scales were; "magnitude scales", where the observer is asked to assign numbers to stimuli in proportion to the magnitude of the stimulus, "partition scales" where the observer assigned a finite set of numbers to each stimulus (e.g. in interval and numeric rating scales) and "confusion scales" that included scales such as JND, discrimination, paired comparisons and successive intervals [168]. A knowledge of these scales also led to a differentiation of the types of measurement such as threshold measurement and suprathreshold measurement. Ocular discomfort is often measured through suprathreshold measurement methods.

Suprathreshold Measurement of Ocular Discomfort:

Many health disciplines are now making an effort to improve the quality of life. If such efforts are to have a sound scientific basis, then the subjective states of a person's well being must be measured in a reproducible and a valid fashion. Discomfort and pain are major markers of the subjective state of a person. According to the ISAP definition, pain is an unpleasant sensory and emotional experience associated with actual or potential tissue

damage, or described in terms of such damage [169-171]. The word "discomfort" is not clearly defined in terms of the sensory or emotional experience. Discomfort is derived from Old French *desconfort* and is defined either as a mental or a bodily distress or something that disturbs one's comfort. The term ocular derived from the Latin *oculus* means relating to the eye and therefore "Ocular discomfort" may be inferred as any mental *or/and* (more appropriately "*and*") bodily distress relating to the eye. Symptoms of ocular discomfort are an inherent part of most anterior ocular surface diseases including the condition of Dry Eye. There is thus an increased need to measure the symptoms of discomfort. The location and duration of pain and/or distress are important aspects of any pain experience, although they are not easily quantifiable. Different types of scales have been employed to measure the sensation of discomfort and pain. Present methods to assess pain and discomfort include Uni-dimensional pain Measurement tools, Multi-dimensional pain measurement tools and Health-Related Quality of Life Measures.

Unidimensional Pain measurement:

The measurement of the intensity of pain as a unidimensional attribute has received considerable attention and many different methods have been developed to measure this sensory attribute. The unidimensional methods are most commonly used for the assessment of discomfort and pain and their use is on the increase. Over a 1000 references were made to 'VAS' and 'Pain' in the 1990s while a simple 'PubMed' search with the same terms reveal that over 500 references have been produced in 2 years between 2000 and 2002 (PubMed Search terms: 'VAS' AND 'Pain', Field: All Fields, Limits: Publication Date from 2000 to 2002, Result: 871) [172]. The unidimensional measurement methods include the categorical verbal rating scales (VRS), the categorical numeric rating scales (NRS) and the visual analog scales (VAS). Other methods of pain measurement are behavior observation scales, and physiologic responses to experimentally induced pain. The VAS, VRS, and the NRS are also used to measure, the relief from pain. Categoric scales assume a special importance in studying ocular discomfort as these rating scales employed traditionally in pain measurement, are often used to characterize ocular surface symptoms and understand their prevalence and impact on the quality of life. Further the diagnosis, severity and classification

of Dry Eye has been made on the basis of such verbal rating scales [126, 131, 132, 159, 173]. Suprathreshold methods of pain measurement are divided into three broad categories.

1) Discrete numerical or verbal category scales.

2) Bounded or confined continuous measure scales such as the VAS.

3) Unbounded scales such as Magnitude Estimation scale.

Discrete Categorical Scales:

These may be discrete numerical (e.g., numbers between 0 – 10) or discrete verbal category scales (e.g., mild, moderate, intense or severe). Categorical variables that have an ordered level are termed as ordinal variables while categorical variables for which the levels do not have a natural ordering are called nominal variables. Sensory and affective pain descriptors can be used to construct ratio scales for discriminating between the sensory intensity and the affect or unpleasantness of pain sensation. Such a 15 point ratio scale that uses sensory and affective descriptors has been constructed to differentiate between the affective and sensory qualities of pain [174, 175]. To understand ocular sensations, verbal category scales are regularly used to assess the magnitude, affective quality and thermal and other attributes of the evoked sensations [176, 177]. Practitioners in the field of tear film/dry eye, most frequently use dry eye questionnaires to evaluate the symptoms of ocular discomfort [178]. These questionnaires include categorical scales that are frequently used in assessing dry eye symptoms to measure the characteristics, prevalence, frequency and severity (including diurnal changes) of the ocular surface symptoms [179, 180]. Problems in using categorical questions are that they are often employed in circumstances where the response is not categorical and in such cases the ignoring of a continuous nature of the response introduces an error into the response along with uncertainty and confusion for the respondent [181]. The second problem with category scales is that there is often a limited choice of response levels. This results in a loss of information and a reduction in the reliability of the scale causing a loss of efficiency of the measurement tool and reduction in its correlation with other measures. It is not fully established whether the NRS or the VAS is more easy to use although the NRS has been shown to be superior in certain instances while in other cases the

VAS and the NRS are believed to be equally sensitive [182-185]. The categorical scales can be analyzed in different ways.

Analysis of discrete category scales with equal distances:

In category scales the categories are considered to be equal steps and assigned integer ranks such as 1, 2, 3, 4, etc. A categorical scale along the method of equal appearing intervals is a one-dimensional scale and assumes that the variable such as pain which we are trying to scale is reasonably a one-dimensional variable. Simple category scales have been used for pain studies such as the combined verbal and numeric rating scale, four point category scale of pain (0-no pain, 1- mild pain, 2- moderate pain, 3- severe pain) or four point category scale of pain relief (none, some, lots, complete) [185-187]. The McGill Pain Questionnaire is also analyzed by assigning integers to the category subscales.

Disadvantages of Category Scales with equal distances:

The major disadvantage lies in the psychometric properties of the scale. It assigns equal distances between the different categories in the response scale resulting in a distortion of the scale. This may result in a consequence where movement from moderate pain to mild pain may seem better than a shift from severe pain to moderate pain. The verbal rating scale categories most often do not have equal intervals and hence the data is ordinal data. This limits the statistical analysis of such data to non-parametric methods. Verbal rating scale is also considered as less sensitive than methods such as the VAS due to the fixed number of response categories. Sensitivity also depends on the number of adjectives used in the scale. If the number of categories used is 11 or more it is considered as sensitive as a visual analog scale. If the verbal rating scale has less than 5 categories then its sensitivity may be reduced [185, 188]. Other limitations are that the scales rely on the patient's ability to read and interpret the words that are mentioned. Very often the patients may not be able to find a word that accurately describes the sensation or reflects the experience. Patients may also feel they lie between two categories. This method is therefore not recommended as the sole method of pain assessment [188]. In spite of these drawbacks due to its simplicity the categorical scales with equal appearing intervals are used extensively. The McMonnie's Dry eye questionnaire is an example of a category scale that has been used to diagnose dry eye with a high sensitivity and specificity [189]. This questionnaire has a grading scheme to

assess the condition of dry eye and some of the psychometric properties of this questionnaire have been documented [189]. The questionnaire differentiated normal subjects from subjects with Sicca syndrome in a defined population. It was originally derived from a literature review analysis of patients with Sicca symptoms, attending a Rheumatology Center. In some of the previous studies employing this questionnaire there were numerous sampling anomalies [190]. The McMonnie's questionnaire consists of forced choice items, with responses ranging from between 3 to 5 points and an arbitrary value assigned to the responses. In two questions no value is assigned to a certain response (Question 5 and 6 of the Dry Eye Questionnaire) [190]. Such questionnaires though widely used may not truly demonstrate the complete properties of the verbal descriptors. To understand the observed relationship between the scale and the attribute, the measurement quality of the scale must be first evaluated along accepted methodologies [191].

Method of Successive Categories:

This is based on Thurstone's law of categorical judgment, where the observer rates the stimulus into a number of ordered categories. The value of each category is determined from the response behavior of subjects simultaneously with the actual scaling of stimulus attributes. In such an analysis the spacing of each category is inversely related to the amount they overlap. The amount of each category is the relative proportion that each response is used to describe the same stimulus [192]. In general the values of a stimulus are measured through techniques of physics and numbers are assigned to sensation magnitudes. The magnitude of the sensory response obtained is plotted against the physical value of the stimulus and a psychophysical magnitude function is obtained. When the physical characteristics of the stimulus cannot be specified, the observers do not report the sensory response directly. A set of equations called the law of categorical judgment is used to scale the stimulus on a psychological continuum. A psychological continuum is a continuum of subjective or psychological magnitudes. Each subjective or psychological magnitude is mediated by a "discriminal process". The discriminal process as defined by Thurstone means "that process by which the organism identifies, distinguishes, or reacts to stimuli". When a stimulus is presented to an observer it gives rise to a discriminal process as the perceptual system encodes the features of the stimulus and converts them into a subjective response.

The discriminal process is affected by the features of the stimulus, the perceptual system of the observer and the sensory system of the observer and these involve both the 'cognitive' and the 'affective' processes of the observer [193]. The result of this process is an impression of the stimulus and its location relative to other possible stimuli. Because of perceptual noise causing momentary fluctuations, a given stimulus is not always associated with the same discriminal process and as a result instead of a single discriminal process there may be a number of discriminal processes for a given stimulus. The discriminal process that is most often associated with the stimulus is the modal discriminal process. The value of the modal discriminal process is the indirect scale value of the stimulus. The standard deviation of the distribution is the discriminal dispersion. The discriminal dispersion and scale values are different for different stimuli. Such procedures of analysis have been applied in the measurement of pain [194, 195].

Disadvantages of the method of Successive Categories:

The method of successive categories often implies that the categories reflect successively more of the stimulus property on the psychological continuum. Another disadvantage is that there is often a loss of both information and precision in information due to the (a priori) categories in the scales.

Determination of Category Values Independently:

The category values of a measurement scale may be determined independently from their use in describing evoked sensation. This is in contrast to the Thurstone method of determination of category values simultaneously with the scaling. The determination of such category values is important because many clinical pain studies cannot provide the stimulus control for a Thurstone's analysis. An example of such an independent determination is the MPQ (McGill Pain Questionnaire). 102 words from the clinical literature were classified into three major classes and 16 subclasses. The three major classes describe the *'sensory qualities'* the *'affective qualities'* and the *'evaluative'* words. Each of the subclasses was given a descriptive label and included words that were qualitatively similar. Subsequently different groups of individuals consisting of physicians, patients and students quantified these words by assigning intensity values to the words. Both the five and seven point numeric scales ranging from least (or mild) to worst (or excruciating), and a Thurstone

analysis was used to determine numeric values for the pain intensities implied by the words. To this classification four more supplementary or miscellaneous subclasses were added resulting in the final classification of 20 subscales with each subscale having two to six items. Some of the MPQ items have been quantified again by a relative magnitude procedure wherein a ratio scaling method was used to rate the relative magnitude of the descriptors. The same ratio scaling method was also used to rate the length of lines that were presented randomly and a psychophysical function was used as a calibration function to convert mean response for each descriptor to a common unit of line length. In this method the results of the quantifications of the verbal descriptors and the line length were converted to a common unit. In other procedures the category values were determined by a cross modality matching to hand grip force and time duration [174, 196]. These methods provide a means to assess the consistency and reliability of verbal category scales. The disadvantage is that they do not address the variability in the absolute magnitude of the words such as how much sensation is represented in the word mild or moderate. This is usually resolved by equating the full range of words across the individuals and by relying on the face validity of the use of a common language. Another approach is to match clinical pain to a pain evoked by a defined stimulus or to match the intensity of pain to a stimulus which is non-painful [197]. Other methods to quantify category values employed procedures where the same subjective scale was used to rate clinical and experimental pain and these studies indicated that the relationship between quantified verbal descriptors and a know stimulus could be described by power functions. The consistencies in the results often support the use of quantified verbal descriptors for the assessment of controlled noxious stimulation and clinical pain [198, 199]. The MPQ has been used to assess ocular discomfort caused by tear drying.

Formats of the Category Scales:

Construction of Scales:

The quantification of verbal descriptors helps in the formation of the categorical scales. Quantified descriptors are used to construct categorical scales which may be *direct estimation techniques, comparative methods or econometric methods.* The general issues involved in the construction of the scales for maximizing precision and for minimizing bias are:

Number of steps needed for construction of the scales: This is determined by considering that if there is less number of levels than the subject's ability to discriminate there will be a loss of information. The minimum number of scale categories should be 5 to 7. Using 5 scale categories reduces the reliability by about 12%. The loss in reliability for 7 and 10 categories is small. A measure to be considered while designing the scale is that raters often tend to avoid the extreme positions of the scale [181, 200]. Discrimination judgments of clinical conditions by experienced observers often have a high inter and intra-observer reliability but this reliability of discrimination may be reduced when the scales contain fewer intervals. While judging conjunctival hyperemia less than 6 intervals reduced the reliability of discrimination. At the same time increasing the number of intervals with the purpose of trying to make the scale fine reduced the concordance between judgments [201]. A finer scale may therefore result only in a moderate improvement of sensitivity. It is shown that for a scale to be moderately sensitive it should not exceed 1 standard deviation (SD) of the discrepancy (discrepancy being the difference between two paired observations A and B; Discrepancy = Observation A – Observation B). For a scale to be fine it should not be less than one third of the SD of the discrepancy. While formulating a qualitative scale the benchmarks defining the intervals should be carefully chosen so that the intervals are evenly spaced [202].

The maximum number of categories that should be used: Discrimination judgement relies on short term memory and while making discrimination judgements an average person's memory has limitations on the amount of information that can be received processed and remembered. The limitations on short term memory is mostly in the order of seven chunks and therefore for practical purposes most scales have an upper limit of 7 categories which should not result in a significant loss of information [203, 204]. Does a scale need an even or an odd number of categories? The research needs dictate this. In bipolar scales the subject may have the choice of expressing no opinion when there is odd number of categories or may be forced to express an opinion if there is even number of categories.

The meaning of the adjectives used in the scales: Many words are used commonly while constructing scales. Verbal qualifiers used to grade the degree of a given attribute may be of the following types:

1) Intensity words such as a little, very, extremely.
2) Frequency words such as never, sometimes, often, always or constantly.
3) Probability words such as hardly, possibly.
4) Quality words such as bad, satisfactory, good, and excellent.
5) Agreement words such as yes, no, sometimes, uncertain, don't accept, agree and true.

These words used to elicit judgements about frequency or intensity may or may not mean the same to subjects and investigators. Subjects often rely on verbal information when the data cannot be easily quantified, but when the data can be quantified, they prefer numerical information [205]. The adjectives used in the scales are in many instances coupled with adverbs that have a probabilistic meaning (i.e. a possibility of a numerical probability or a range of probabilities corresponding to the phrase may be present) or terms which indicate the frequency (e.g. very, quite, 'no adverb', rather, fairly, somewhat). When using these words, there is often 'between-subject' variability in the numerical values assigned to the terms. Such results indicating variability have also been replicated even when experts who used verbal descriptions in their work were subjects. The high between-subject differences are thought to be due to individual differences in language usage and context effects [204, 206, 207]. The within-subject variability is not minor, but is considerably less than between-subject variability and within a particular context subjects are often consistent in the interpretation of verbal expressions [204]. There is also an enormous overlap among the terms used. The probability of a "highly probable" event ranged from 0.60 to 0.99 with variability in other phrases, including an overlap of 0.2 between terms like, unlikely and likely [206]. Other verbal expressions (e.g. always, never) are interpreted consistently among subjects and may be used as *'anchor phrases'* [208]. The use of verbal expressions is not inferior to numerical estimations and it is important to select a reduced set of phrases whose ordinal properties are generally agreed upon and which could be used with little or no confusion. A serious restriction of the vocabulary which may reduce the discriminating

power must be avoided and a careful selection of the words that are sufficiently apart of each other, will eliminate this overlap greatly [204]. The Melbourne Visual Impairment Project which is a population based study used the ratings of absent/none, mild, moderate, and severe. For each of these words the definitions were provided which specified the investigators definitions of these levels [132]. Descriptors are also used in clinical grading scales and in spite of the continuous development of various methods to assess clinical conditions objectively, the subjective grading scales have shown good repeatability and will continue to be used [209].

Numbers placed under words: When numbers are placed under the words, with a negative to a positive continuum it is often construed to have a bipolar conceptualization of the attribute of interest, whereas the presence of only positive numbers may give rise to a unipolar conceptualization. The numbers often make a difference in the interpretation of the words. Numbers have been placed under the words with the assumption that ordinal variables can be treated as conforming to interval scales. The advantage of treating ordinal variables as interval variables is that it permits interpretable statistics and this provides an increased understanding of the characteristics of the data [210, 211].

Data may be ordinal or not ordinal in nature: An issue with rating scales is that they are on an ordinal level of measurement. The distance between the successive categories may or may not be the same, but unless the distribution is skewed, the data from rating scales are often analyzed as if they are interval data. In the event there are non-equal values these are determined indirectly by the variability between the category choices [192].

Direct Estimation techniques:

The direct estimation techniques require the subject to indicate by a line or a check in a box, an estimate of the magnitude that is being measured. The adjectival scale measurement may be done by a discrete responses or continuous responses. The rating scale with a continuous response is also termed as the graphic rating scale. This is similar to the VAS which is also a direct estimation method. The Likert scale is an example of a direct estimation scale where the subject has to express opinion by rating agreement with a series of statements [212, 213]. The responses are usually on a continuum of agree-disagree. Mainly four important

principles are followed in the construction of the Likert-type scale [214]. These principles are:

1) Isomorphism; This consists of identifying the goal of the assessment to have an isomorphism (i.e. a conceptual and structural consistency) between the construct and the way it is being measured.

2) Singularity; this means that each of the statements on the scale should have only one idea or else it will not be possible to ascertain the level of agreement with an item.

3) Social desirability management; indicates that statements which are socially appropriate or inappropriate should be avoided as the level of the agreement being assessed will reflect more what the respondents think the surveyors want to know.

4) Knowledge liability; means the response should be the reflection of actual opinion and should not be dependant on facts for an accurate appraisal.

Likert scales having between 3 to 11 categories have been used to diagnose and assess the frequency and severity of ocular symptoms due to various causes and their impact on the quality of life [126, 190, 215-219]. In a study rating the handling of contact lenses, the 5 point Likert scale was compared to other scaling techniques and was found to be the least satisfactory scale while in another study the seven point Likert scale was not statistically different from the VAS and it showed a comparable responsiveness with advantages such as easy interpretation and administration [220, 221]. Another widely used method of direct estimation is the *semantic differential scale or the Osgood's method* [222]. The technique developed to deal with emotions and feelings is based on the idea that people think dichotomously. The method is a development of the Likert scale and adds three major dimensions of judgment which are the evaluative factor (e.g. good – bad), the potency factor (e.g. strong – weak) and the activity factor (e.g. tense – relaxed). These EPA ratings usually consist of a 5 or 7 point bipolar scales although any number of scales can be used. The intention is to obtain more information about the attribute being measured by understanding the links between the attitudes and behavior. The advantages of these scales are that they are simple, economical and can be used in most circumstances and are valid in cross-cultural comparisons [223]. The EPA measurements are appropriate when one is interested in

affective responses. A disadvantage associated with these methods of measurement is that it is assumed, the adjectives chosen mean the same to all the subjects. Another disadvantage is that the correlation between the stated attitudes and the actual perception of a subject is often low as it is shown that attitudes are poor predictors of behavior and actions [224].

Direct estimation methods all have the advantage that they are easy to design and administer. They require less pre-testing as compared to the comparative method and are easily understood by the subjects. An important disadvantage is that bias with the 'halo phenomenon' is often noted and this may result as the intent of the questions is known to the subject as well as to the researcher.

Comparative Methods:

The comparative methods have the quality of being able to transform the rank order data or comparative preference data into an interval scale. Such an approach addresses problems associated with the ordinal nature of unidimensional rating scales. The comparative methods are an advancement of the Thurstone's scaling of psychological stimuli [192]. The original three methods of Thurstone's scaling (i.e. paired comparisons, successive intervals, and equal appearing intervals) are not widely employed due to a number of limitations but modifications of these scaling methods are often used [225-227]. Comparative methods commonly used in a modified way are: Thurstone's method of equal appearing intervals, Guttman scaling and the paired-comparison technique.

Thurstone's method of equal appearing intervals:

A large number of attitude statements spanning the range of all possible options, regarding the attribute/attitude being assessed are generated. The large number of statements or 'scale items' are rated on a 1-to-11 scale (1= least favorable to concept, 11= most favorable to concept) and the median rank and the interquartile range is computed (the interquartile range is the difference between the 75^{th} percentile and the 25^{th} percentile). The selection of the items for the scale is then done by sorting the items in ascending order by median and within that by descending order by interquartile range. The statements selected are those with the smallest interquartile as these have the least variability across the judges. The items span the entire range of values. These items comprise the scale and the respondent's score is the

average score of the items selected. The principles of this method are that the scoring of items should be linear for analysis, the items should be 'sample free' and should not depend on whose responses they were estimated from, they should be 'test free' and should not depend on which items they were estimated from, the missing data should not matter, and the method must be easy to apply.

Guttmann Scaling:

Guttmann scaling in pain measurement is generally used to determine a decline or change in the functional ability/disability due to pain, progressive deteriorations of a disease state, effect of different measures on the management of disease state, and to assess global health status of patient populations [228-230]. Guttmann scaling is similar to Thurstone's scaling method. It specifically addresses only a single underlying attribute. The scale is created by generating a large sample of items that reflect the attribute being assessed. The items are administered to a group of respondents, who rate (by agreement or disagreement with a set of attitudes) how the item is related to the attribute or construct of interest. Following this, a matrix or table is constructed showing the responses of all respondents. Two steps are involved in the construction of the matrix (scalogram). The items are ranked in an order of extremes with the most extreme being placed first and other items are placed in decreasing order of extremeness. The respondents are placed in the order of favorableness with the most favorable being placed first followed by a decreasing order of favorableness. This helps to determine, from the total pool the cumulative value (frequency and score) of each of the items that approximate the property of the attribute. Each item now has a scale value obtained from the cumulative analysis. The final scale items are then administered to a subject and the subject's scale score is computed by adding the scale values of every item they agree with. The degree of approximation of the score to perfection is measured by the coefficient of reproducibility, which should be higher than 0.9 in most cases for the scale to be considered unidimensional. Advantages of the Guttmann scales are that a single number carries complete information about the exact pattern of responses to every item. It measures for reproducibility and scalability. The disadvantage is that unidimensional domains are rare.

Paired Comparison Technique:

The paired comparison method is based on Thurstone's law of comparative judgment. In this method each stimulus is paired with each other stimuli and with 'n' stimuli there are 'n (n-1)/2' pairs. This is similar to the method of equal intervals but the two methods differ in approach to calibration. While in the method of equal intervals the items are ranked, in the paired comparison method each of the items are compared to each of the other items and a judgment about which of the two has more of the property is made. Data indicating the proportion of times each option is chosen over the other is displayed in a matrix. The table is then converted into z scores to assign weights to each item. This scale with weighted items is administered to subjects and the final score is the sum or average for all the items, as these items are interval level measurements. This method is appropriate only when a few items are to be scaled. Paired comparison techniques are often used to compare between two lenses or eye drops and such methods may indicate the efficacy of one treatment method over the other [231].

All the three comparative methods require more time for development as compared to direct scaling methods. A clinical scale that has used such a procedure in both acute and chronic pain measurement is the Descriptor Differential Scale. An advantage with the comparison methods is that they provide a measure of the scaling consistency and indicate subjects who do not attend the scaling task. This aids in eliminating uncooperative subjects and helps to improve the psychometric properties of this rating scale [232-234]. Multiple items also permit the use of different alternative questionnaires with each questionnaire having different descriptor items that are theoretically equal in value. This is helpful to rate the pain over a period of time as the use of the same descriptors repeatedly may result in a rating with memory of the previously used pain descriptors. The average of multiple items reduces the scaling errors and these averages are considered superior to single responses or to the mean of only a few responses [235]. Randomization of the categories reduces the chance of the item being chosen on the basis of location, and forces choice on the basis of content.

Continuous Measure Scale:

A continuous measure scale may be bounded or unbounded.

Bounded Continuous Scale:

The visual analog scale is the most commonly used bounded continuous scale. It is considered by many to be a simple measurement tool and is frequently used for assessing the variations in the intensity of pain. The VAS is considered the "gold standard" for assessing clinical and suprathreshold pain by some researchers while others strongly discourage the use of the VAS to rationalize inappropriate procedures especially in the absence of objective symptoms [236, 237]. The VAS is also used in assessing the effects of treatments. It is typically a 100 mm scale that is continuous and is often independent from language (except when anchor words are used). The VAS has a large number of response categories and is more sensitive to changes in pain intensity although in practice the VAS is considered valid for detecting fine changes with a fewer pain levels [238]. It is widely accepted that the VAS has ratio scale properties and therefore the data obtained from the VAS is analyzed by parametric methods of statistical analysis. The most widely used intensity scale for palliative pain, is the verbal rating scale incorporating the terms "none, mild, moderate, and severe" at intermediate positions and a substantial correlation has been demonstrated between this VRS and the VAS [239, 240]. In one study the terms "moderate" pain and "severe" pain were correlated with corresponding VAS scores and a baseline VAS score in excess of 30 mm was considered as moderate pain with a mean score of 49 mm and corresponding severe pain was 54 mm with a mean score of 75 mm [241]. The advantages of the VAS are that the scores of the VAS have the qualities of ratio data provided the data are normally distributed [188, 242-244]. The disadvantage of the VAS is that the attribute of interest is measured on a single scale and this is overcome by using multiple visual analog scales to assess the related aspects of an attribute of interest. The contribution of the various dimensions of pain to the ratings is not known. If the ends of the VAS are labeled either by words or numbers, it affects the ratings of the attribute of interest. Other disadvantages are that subjects or patients especially the elderly may not find it simple and often find it difficult to complete the scale. The non-compliance rates range from 7-26% [182, 183, 245-248]. Reliable ratings cannot be produced across different groups of patients as patients may interpret the scale differently [172, 249]. There is also a poor reproducibility of the scale with those who have a cognitive dysfunction, patients in immediate post-operative period and in those suffering from dementia [250, 251]. Despite these limitations the VAS is favored because it places a

minimal demand on the ill patient and because of its simplicity. Some of the limitations of the visual analog scale have been overcome by the visual analog thermometer (VAT) which was used initially in the pain rating of patients suffering from burns. The VAT has shown a close correlation with the standard visual analog scale and the numeric rating scale [252]. Many other modifications of the VAS exist but the psychometric properties of these instruments have not been fully explored and therefore it is difficult to arrive at conclusions regarding their performance. In the measurement of ocular discomfort, the VAS has been widely used lending a reliability and validity to its use in the measurement of symptoms of ocular dryness and ocular irritation [253]. While employing continuous scales in clinical observations it is important to develop confidence limits so as to distinguish between true and observed findings (any observation score O = T + E + B, where O is the observation score, T = True Score, E = Random Error and B is the Bias). The limits are often set at a 95% confidence interval and in general it is desirable to have a narrow confidence interval. Visual analog scales are frequently used to assess the symptoms of ocular discomfort [220, 254].

The Unbounded continuous scales:

Ratio scaling procedures constitute unbounded continuous scales and are of four types. These are ratio production, ratio estimation, magnitude estimation and magnitude production. The most common method used in pain measurement is the method of magnitude estimation which has shown accurate ratio responses, with a reduced between-subject and within-subject variability [255, 256]. All the ratio scales have some common characteristics.

Before using ratio scales instructions need to be given to subjects for evaluating and responding in terms of ratio or judgements regarding proportion. These instructions may include a modulus (where a specific number is chosen for the first response) or they may be modulus free allowing any number to be chosen for the first response.

All the ratio scaling methods theoretically offer unlimited continuous response. This is especially true for the methods involving magnitude estimation (numbers), and time duration. Other unbounded ratio methods may in actual practice be bounded due to mechanical, physical or safety limitations.

Ratio scales assume the power relation between stimulus and the response. The relation between the stimulus intensity and the response modality is described by a power function of the form $\psi = k (\phi - \phi_o)^n$, where ψ is the subjective magnitude that grows, as ϕ the stimulus magnitude is raised to the power of 'n', and ϕ_o is the effective threshold (also termed the threshold correction), and k is a constant. From the psychophysical function of each different stimulus modality, its specific exponent has been derived as the ratio of response-specific function to stimulus-specific function [257]. In magnitude estimation the numbers are used to describe sensations and this has been arbitrarily assigned an exponent of 1. Hence any stimulus modality judged with numbers as in magnitude estimation, will have an exponent which is the reciprocal of the exponent of the psychophysical function. There are two methods of applying magnitude estimation in pain studies. In the modulus dependant method, the observer is presented a standard stimulus which has a certain modulus (numerical value) and when stimuli are presented subsequently the observer assigns numbers to each of the stimuli with a value that is relative to the value of the modulus. The data from several observers is combined and the median or the geometric mean can be calculated for each stimulus value. In the modulus free method the modulus is not assigned by the experimenter. The subject establishes his or her own modulus and the stimuli presented are assigned numbers in proportion to their magnitudes as perceived by the subject. The modulus free method is often preferred as the observer is permitted to choose his or her modulus. In both of the above methods the psychophysical magnitude function is the average magnitude estimation plotted as a function of a property of the stimulus. This method of pain measurement has the following advantages. No extensive training is needed, judgments can be obtained rapidly and several parameters of the stimulus can be studied extensively.

The fourth characteristic of ratio scale procedure is that stimulus spacing should be small to cause confusion regarding the identity of the stimulus and the number of stimuli so that subjects do not identify the stimulus to give same response to an identified stimulus. Factors which can influence the exponent include the value of the threshold correction, stimulus and response range (a small stimulus range employed frequently while delivering painful stimuli) increases the value of the exponent and a constricted response scale reduces the

exponent's value [258]. A lowered exponent is often termed as virtual exponent as it reflects the compression effect on the top of the scale along with the unrestrained continuum [259]. The value of the exponent also depends on the instructions given to the subjects and with appropriate instructions and adequate training the compressive effect on the exponent was found to diminish [260, 261]. The power functions that are derived from the psychophysical function are now being used to describe a neural response relation. In one study on the basis of parallelism between, psychophysical response to CO_2 stimulation of the human cornea and the CO_2 concentration-firing frequency response curve for single unit activity of polymodal nociceptors in the cat, it was inferred that pain sensations evoked in the human cornea, by CO_2 stimulation was due to excitation of polymodal nociceptors. Further, the power function exponents derived from CO_2 concentration-pain sensation curve of human cornea was found to be similar to exponent values obtained by CO_2 stimulation of the nasal mucosa leading to the inference that similarities could be due to the protective role of the nociceptive system [262-264]. Another use of power functions is that when different types of cross modality methods are employed to derive similar power function exponents in the study of qualitative pain, it may provide a way to quantify the qualitative descriptors of pain intensity [265, 266]. The slopes (exponent) of the psychophysical functions also permit a comparison between different sessions to understand the reproducibility of pain and methods can be devised to minimize the between session variability [267]. The method of line production is another cross modality matching method where the subjects draw a line of any length to indicate the amount of pain perceived.

Other Unidimensional Suprathreshold Scaling techniques:

Some of the other methods of suprathreshold scaling are the methods of discrimination and the method of stimulus integration. These response dependant methods consist of an S x R stimulus matrix where the stimulus intensity S is presented R number of times. The response method may be direct scaling using Verbal descriptors, categorical scales, VAS or cross modality matching. A modification of the sensory decision theory task (mostly used in threshold detection of pain) is also employed. Many sets of four stimulus intensities are presented and subjects use a 12-14 point category scale to assess the pain. These methods are used only by a few people in the assessment of pain [268]. The above mentioned

methods measure mainly the intensity of pain sensation; the unpleasantness of pain is commonly measured with the use of language. Scales which measure the affective dimension are the hedonic pain scales.

The Hedonic Pain Scales:

The affective dimension of pain includes the hedonic aspect of pain which is the degree of unpleasantness. With gustatory and olfactory senses the hedonic nature of the sensation may be either pleasant or unpleasant. In pain the hedonic nature is only unpleasant or disagreeable and this makes the evaluation difficult. As the hedonic pain is more qualitative in nature language is used to evaluate the affective dimension of pain. Words describing pain are categorized and quantified and the use of these words in different studies has indicated a consistency and generalness in the structure of affective descriptors for measuring the affective dimension of pain [269, 270]. In addition to the intensity and unpleasant quality of pain, there are other qualities such as burning, squeezing, and throbbing. The evaluation of these qualities of pain and the similarities within them is done by the process of multidimensional scaling.

Multidimensional scaling:

The method of multidimensional scaling is considered useful especially to understand the different dimensions of pain. Multidimensional scale is considered useful if the following considerations are fulfilled:

1) It should lead to an increase in the accuracy of the pain measurement which is determined by the reliability of the measurement method.
2) It should aid in the diagnostic accuracy of the condition.
3) It should provide the researcher or clinician a greater understanding about the pain of the subject.
4) It should provide an understanding of the relationship between psychophysical data and neurophysiological causes of pain.

The raw data for the multidimensional pain is collected by the methods of verbal descriptors, visual analog scale, and other cross modality matching methods described above and

through physiological responses. The verbal descriptors do contain subtle differences and lend themselves to a better analysis. The dimensions that are usually latent are revealed in the analysis of the data, and are generally not directly observable. It provides a spatial representation of the data and helps to facilitate the interpretation of the data. The two types of multidimensional scaling methods are metric and non-metric models. The four basic steps of MDS include; data collection to form the similarity/dissimilarity matrix, extraction of stimulus coordinates, determination of the number of coordinates representing the data, and rotation and interpretation of the data. A few of the common methods of analysis used in pain studies are factor analysis, ideal-type analysis and INDSCAL (Individual differences scaling). Symptoms of ocular comfort have been studied through the method of MDS and factor analysis revealed the different dimensions of groups of ocular symptoms associated with symptoms of contact lens wear [271]. In evaluating discomfort induced by ophthalmic drops, a two dimensional space accounted for most of the symptoms with burning/stinging in one dimension and oily/slippery in the other dimension [272]. Multidimensional analysis of ocular comfort between post menopausal symptomatic group and an asymptomatic normal population revealed that despite the presence of symptoms of dryness in one group, the ocular comfort was scaled in a similar way by both the groups [273]. This revealed that comfort and dryness could be viewed in different orthogonal dimensions in the multidimensional derived space. Such methods help to define a set of independent dimensions with high internal consistency. The utility of factor analysis was demonstrated during the assessment of dry eye in a large population based sample [158]. With the diagnostic techniques improving and with the imaging of pain and discomfort becoming more sophisticated, the importance of multidimensional scaling will increase. A summary of the data collection methods, analysis and advantages and disadvantages of multidimensional scaling are mentioned in table 4-1.

Some of the other methods of measuring discomfort and pain especially in subjects who do not have language skills are through observations of behavior. There is however a very low concordance between the ratings of pain by subjects and by medically trained personnel. Whenever there is such a discrepancy existing, there is often a tendency to overlook the report of the patient or subject. These issues should be resolved by taking multiple measures

of pain bearing in mind that pain is defined as a "subjective experience" and self reports are the most valid methods of assessing pain and discomfort.

Computerized method of Pain assessment:

Computers are now widely used in devising methods to test discomfort and pain. Electronic dairies with questions displayed permit the patients/subjects to enter categorical or numerical data for evaluation of discomfort and pain. The use of such instruments is increasing [274-277]. Computerized numeric rating scales with touch screen have made the immediate evaluation of a questionnaire possible [278]. Electronic visual analog scales have been developed and their use is on the increase [279, 280]. Any design process that involves the development of an electronic pain measurement tool broadly consists of the following basic steps. It consists of an 'Input' where the subject's registration and demographic information is present. The input information is available to the other components of the application and is used to sort the results and generate the reports. The 'Stimulus' part of the application enables the subject and the experimenter to view the stimulus on the screen. The stimulus may be provided through an audio, video, live interaction or through complex programming. Having the stimulus incorporated into the application has the advantage that the administration of the test is made simple. The 'Data collection' component enables the subject and the experimenter to enter responses to the assessment items. Subjects enter information where there is a stimulus component. The experimenter may enter behavioral observations. This is followed by the 'Processing' stage of the application where the unprocessed responses are loaded into an analysis program. The 'Reporting' displays the results on the screen. Each of the components should be tested before it is put to actual use [281]. The computerized methods have been found to be useful for measuring the temporal characteristics of pain which are included in the IASP taxonomy of pain. According to this taxonomy pain can be (1) Continuous or near continuous and non-fluctuating; (2) Continuous or nearly continuous and fluctuating; (3) recurring, irregularly; (4) recurring, regularly; (5) paroxysmal; or it may be (6) sustained with superimposed paroxysms [282]. The fluctuations of pain have not been studied much and most conventional methods of pain measurement may not have the ability to record acute fluctuations occurring over a short

interval of time. The computerized technologies have a tremendous potential in the measurement of pain and discomfort.

The above review of the techniques for the suprathreshold measurement of ocular discomfort is only a brief summary of the vast literature available about ocular discomfort. A better understanding of the measurement of ocular discomfort and pain is possible only when it is coupled with knowledge of the physiologic mechanisms responsible for the maintenance of a healthy ocular surface.

These mechanisms will be considered in the next section.

Multi-dimensional Measurement - Metric or Non-metric		
Common data collection methods	**Analysis methods / Advantages**	**Disadvantages**
▪ Categorical Verbal Rating Scales (more nuances of stimulus judgements) ▪ Categorical Numeric Rating Scales (Greater Sensitivity scale employed) ▪ Visual Analog Scales (a 10cm line either horizontal or vertical) ▪ Cross modality matching methods, ▪ Paired comparisons ▪ Physiologic responses ▪ Observed behavior	▪ Common methods of analysis are • Factor Analysis • Ideal-type analysis • e.g. INDSCAL ▪ Increases reliability or accuracy of pain measurement ▪ Accurately characterizes subtle changes in pain (rotational quality) ▪ Projection methods enable data to be visualized along different orthogonal dimensions in MDS space ▪ Projections help discover independent dimensions ▪ Independent dimensions can be often defined with high internal consistency	▪ Often a great increase in amount of raw data collected ▪ The number and quality of the different dimensions are highly situation and method specific ▪ Dimensions may be difficult to interpret ▪ In clinical situations refined analysis of pain quality may not increase accuracy of pain rating ▪ Application to clinical situation is often not easy

Table 4-1: Summary of the Advantages and Disadvantages of Multidimensional Scaling.

5. Structure and function of the Tear Film:

The structure of the tear film has been extensively studied to contribute to the understanding of the ocular surface. The morphology of the tear film is dependant upon a normal lid structure and lid closure [283] and the state of the ocular functional unit. Normally there is a continuous alteration in the constituents of the tear film due to the constantly changing climatic and environmental influences [284]. The tear film was believed to be a three layered structure consisting of a mucous layer close to the epithelium, an aqueous layer outside of the mucous layer and a lipid layer on the external surface of the aqueous layer [285]. The present concept is that the tear film is a bilayered structure consisting of an aqueous/mucinous phase and a lipid phase [286]. The mucous layer has a refractive index that is identical with the aqueous phase of the tear film and hence is difficult to visualize [287]. The aqueous layer and the lipid layer are easier to visualize.

The lipid layer of the tear film:

The lipid layer is the outermost layer of the tear film. It consists of an outer non-polar lipid layer with anti-evaporative properties and an inner polar layer with surfactant properties.

The three essential functions of the lipid layer are:

1) To provide an effective barrier and prevent evaporation.
2) To provide a surfactant layer that acts as an effective bridge between the non-polar lipid layer and the aqueous mucinous layer [288].
3) To maintain compression and expansion of the lipid film without hysteresis as the eye blinks to prevent tear overflow [289].

Other functions of the lipid layer are:

4) To prevent the maceration of the skin lid margin by the tear. To form a barrier for preventing contamination of the tear film.
5) To prevent the spreading of sebum from the cutaneous glands into the eye.
6) To provide a smooth surface for refraction of the incoming rays of light [289, 290].

For the most part the lipid layer is formed by the secretion of the meibomian glands. A small contribution to its formation is from the glands of Zeis.

Morphology of the meibomian gland:

The meibomian glands are compound tubulo-alveolar holocrine glands, which secrete the meibomian oil forming the lipid layer of the tear film. There are about 25 glands in the upper lid and about 20 glands in the lower lid. These glands are arranged in a single row along and perpendicular to the lid margin. Each gland has a long central duct surrounded by acini. Saccular acini project from the main tubules of the glands. The acini, which may be single or composite, consist of a layer of flattened basal cells resting on a basement membrane. This outer cell layer of the alveoli forms the germinal layer. A cell differentiation in the centripetal direction results in an enlargement of the cells, loss of nuclei, lipid accumulation, and degeneration of the centrally located cells that forms the holocrine secretion. The basement membrane of the acini separates the gland from the surrounding lymph space and the stroma of the tarsal plate. The tubules of the acini join to form the meibomian duct. Four layers of cells line the duct of the gland and at the region close to the orifice there may be six layers of cells. The orifices of the glands emerge anterior to the mucocutaneous junction, through punctal openings and consist of three concentric rings. The rings are described as an inner opaque cuff, followed by a dark or translucent middle opaque ring, and an outer opaque cuff, which surrounds the punctum of the meibomian gland [290, 291]. Dense collagen, fibroblasts and a network of blood vessels and nonmyelinated nerve fibers surround the glands. Elastic tissue and smooth muscle fibers are found around the acini. In the meibomian glands of the primates the nerve fibers are mainly associated with the acinar structures. The stimuli for the secretion of the meibomian glands have not been clearly identified. The nerve supply of the meibomian gland and their role in meibomian secretion is described below.

Regulation of the lipid layer secretion:

The secretions of the meibomian gland mainly contribute to the formation of the lipid layer of the tear film. The glands are innervated by the sensory nerves, the sympathetic and the parasympathetic nerves. Different neuropeptides have been identified by specific antisera in the human meibomian gland. These include substance P (SP), the calcitonin gene related

peptide (CCRP) and the vasoactive intestinal polypeptide (VIP). The neuropeptides, neuropeptide Y (NPY) and the neuronal enzyme tyrosine hydroxylase (TH) have been identified in the meibomian gland of primates, rabbits and rats [292-294]. In the cynomolgus monkey the acini of the meibomian gland appear to be surrounded by a mesh of unmyelinated nerves and terminal axons with varicose endings. These varicose axons contain small agranular and large granular vesicles and show an immunoreactivity to the neurotransmitters, neuropeptide Y and the vasoactive intestinal polypeptide (VIP) indicating a parasympathetic nature of innervation [295]. The proximity of the nerve fibers to the acini of the meibomian gland and their immunoreactivity to the peptides of the autonomic nervous system indicate a possible neurotransmitter mechanism for the modulation of meibomian gland function [296]. In the adult rats a parasympathetic neuronal regulation of the meibomian gland has been demonstrated [297]. Using retrograde tracers, the sensory and sympathetic CGRP immunoreactive nerve fibers were traced to neurons located in the trigeminal ganglion and the superior cervical ganglion [298, 299]. It is believed that CGRP causes a vasodilation in the ocular tissues with a consequent increase in the level of the circulating hormones leading to an increased secretion of the meibomian gland. These nerves and circulating hormones are believed to regulate the meibomian secretion. This regulation of meibomian secretion may be controlled at different levels including; (a) a regulation at the level of lipid formation (b) a regulation of the rate of maturation of the holocrine mechanism in the meibomian gland and (c) a regulation of the rate of release of the meibomian secretion. There is increasing evidence that the meibomian gland is under a hormonal regulation [292, 300]. The presence of estrogen and progesterone receptor mRNAs in the meibomian gland, lacrimal gland acinar epithelial cells, lid, palpebral and bulbar conjunctiva and the cornea have been established immunohistologically. Androgen receptor proteins have been demonstrated in the acinar epithelium of the meibomian gland, epithelial cells of the human lacrimal gland, the bulbar and forniceal conjunctivae, cornea, the lens and the RPE cells [301, 302]. These target sites of androgen activity possess the ability to locally convert testosterone to dihydroepiandrosterone (DHEA). In the skin an increase of DHEA leads to an increase in the sebum secretion, while a decrease in DHEA causes decreased sebum secretion [303]. In the eye it has been demonstrated that anti androgen medications are associated with meibomian dysfunction and an unstable tear film resulting in symptoms

of dry eye [304]. The lipid secretion in the androgen target organs is therefore related to the levels of the circulating androgens and estrogens, and this relation is especially prominent in the aging individuals. An endocrinosenescence due to aging results in a decline of several hormones. Changes are especially seen in the adrenal and the gonadal glands where there is a decline of serum/plasma hormones such as DHEA, DHEA sulfate, 17 β-estradiol and progesterone [305, 306]. The deficiency of the circulating androgens and estrogens leads to meibomian gland dysfunction resulting in tear lipid profile changes that may cause an evaporative type of dry eye [300, 307]. The regulation of the meibomian gland therefore seems to be dependant on both neurotransmitters as well as hormones. The relative influence of each is not presently understood though it is possible that the lack of hormonal support may play an important role in the aging individuals.

Composition and formation of the Lipid Layer:

There is a large variation in the tear lipid content in the normal individuals. The main constituents of the tear lipid are the wax esters, sterol esters, polar lipids, diesters, triglycerides, free sterols and the free fatty acids.

Liquid composition of Human Meibum	Percentage
Cholesterol Esters	29.5%-38.0% (or less)
Wax Esters	35.0%-47.0% (or more)
Triglycerides	29.5%-38.0% (or less)
Polar Lipids	6.0%-16.0%
Diesters	2.0%-8.4%
Free fatty acids	2.1%-2.5% (or less)
Free Cholesterol	1.50%-1.80%
Hydrocarbons	3%-7%

Table 5-1: Composition of the lipid layer (Data source [290, 308-310])

The lipids of the tear film, like thin films in the other biological systems form a lipid monolayer [308, 311-313]. At present very little is known about the mechanism of in-vivo formation of ocular film lipid monolayers but it is observed that the phospholipids form aggregates including sheet-like monolayer structures. Our knowledge regarding the

dissolution of these layers and their implications in evaporative dry eye disease of the ocular surface is even more limited [2]. *In vitro,* the formation of the lipid monolayer requires:

1) Negatively charged lipids (as even a reduced quantity of the negatively charged lipids leads to a multilayered formation) [314].
2) A small quantity of protein which aids in the formation of the monolayer.
3) An adequate concentration of monovalent ions (sodium less than 10mM) and divalent ions (calcium less than 1mM) to aid the formation of monolayers.

In the tear film, a lipid monolayer establishes itself as the above conditions are satisfied. The meibum contains anionic phospholipids such as phosphatidylserine (7%), phosphatidylinositol and cardiolipin [315, 316]. The aqueous layer of the tear film contains about 150mM of univalent ions mainly sodium and potassium and 1mM of divalent ions mostly calcium and magnesium. Small amounts of protein are also present.

The normal appearance of the lipid layer of the eye has been studied using non-invasive methods such as Interferometry and specular reflection. Based on its thickness and appearance the lipid layer is classified as having various patterns such as Open meshwork, Tight meshwork, Meshwork and wave, Wave appearance, Amorphous, and Lipid layer with 1st order interference colors [317].

Brewster angle microscopy provides a direct observation of the meibomian lipid layer following spreading in a Langmuir type trough. The lipid layer thickness ranges between 45 nm to 135 nm [318]. In the *in vitro* studies, the normal lipid layer is observed to be homogenous and mobile at a surface tension of 5.0mN/m, with areas of lower and higher reflectivity. The lower reflectivity lipids are 2nm thick and higher reflectivity lipids are 8-10 nm thick.

Lipids from healthy persons may be inhomogeneous and mobile whereas in conditions such as meibomitis, the lipid layer is inhomogeneous and immobile [311]. Based on the appearance, the lipid layers have been classified into normal and abnormal. The common causes for abnormal lipid layer are meibomian over secretion, an abnormal meibomian secretion due to disease or inflammation, abnormal spreading of the lipid layer, rapid lipid break-up, natural contamination, effects of cosmetics, eye drops and contact lenses. Soft contact lenses cause the lipid layer to be thinner [317]. In the case of rigid contact lenses, the

Classification of Abnormal tear lipid layer appearance		
Pattern	**Description**	**Observation**
Meibum Over-secretion	Large thickness variation	2^{nd}, 3^{rd} and 4^{th} order interference
Abnormal Secretion	Thin lipid layer due to undersecretion	Hardly visible
Blepharitis	Irregular and globular	Good appearance
Abnormal Spreading	Variable thickness and thinner in upper region	Well apparent in incomplete blink
Poor Mixing of the newly formed lipids	Secretions take longer to come to a stop following blinking.	Surface plaques or streaks or oily lenses
	Lipid island formation with high tear evaporation	Grey area separated by zones of high reflective lipid cover

Table 5-2: Classification of abnormal lipid layer (Data Source [317])

Grades of Lipid layer Interference Pattern		
Grade	**Observation**	**Type of eye**
Grade 1	Somewhat grey color, uniform distribution	Normal eye
Grade 2	Somewhat grey color, non-uniform distribution	Normal or Dry Eye
Grade 3	A few colors, non-uniform distribution	Dry eye
Grade 4	Many colors, non-uniform distribution	Dry eye
Grade 5	Corneal Surface partially exposed	Dry Eye

Table 5-3: Grades of lipid interference pattern (Data Source [319])

lipid layer is more often very thin or absent. Another classification based on the tear lipid layer interference pattern differentiates the lipid layer into 5 grades. A significant correlation between the interference pattern, the lipid layer appearance and other dry eye examination modalities such as fluorescein staining, schirmer's test, rose bengal and tear film breakup time was present. The 5 different grades of Lipid layer interference based on the appearance are mentioned in Table 5-3. Other modes to differentiate the lipid layers exist [318, 320].

Functions of the Lipid Layer:

The three important functions of the tear lipid layer are:

The Prevention of Evaporation:

In humans the lipid layer reduces aqueous evaporation by about 90%-95% [321]. In the absence of the lipid layer the rate of evaporation may increase four-fold [322]. A ten to twenty fold increase in evaporation from the corneal surface of rabbits is noted when the lipid layer is removed. The lipid layer consists of two phases. The outer and thicker phase termed as the non-polar phase, retards the transmission of water vapor and increases the resistance to evaporation. This phase is believed to be dependent on the structure of the inner polar phase [289]. The deep polar phase comprising of surfactant phospholipids is a bridge between the aqueous and the non-polar lipid phase. The surfactant property of the polar phase and the barrier function of the non-polar phase could be due to their specific compositions. When lipid layers consist of closely packed parallel hydrocarbon chains, the resistance to evaporation is increased because the energy barrier to the diffusion of the water molecule across the lipid chain is very high. This is even more pronounced if the lipid chain is long, and it is suggested that the resistance\chain-length relationship for monomolecular layers is an exponential relationship [323]. Normally about 10% of the total tear volume evaporates, while 90% drains through the lacrimal punctum [324]. An increased aqueous evaporation is due to a poor quality lipid layer being associated with lower tear film stability and regardless of the thickness a stable lipid layer retards the evaporation of tears [322]. The presence of lipid deficiency alters the evaporation rate with a 1.5 to a 3.0 fold increase in the evaporation rate of tears. When aqueous deficiency is combined with a meibomian gland dropout, there is often a four fold increase in the rate of tear evaporation. This leads to a high tear osmolarity and a decreased tear break up time leading to a vicious circle of increased

evaporation and ocular surface damage [290]. In meibomian gland disorders, the tear electrolytes increase uniformly, while in the lacrimal gland disease, the Na+ rises secondary to an increased secretion caused by the low flow rates [325].

Surface Spreading Function:

The surface spreading function of the lipid layer is important for the establishment of the tear film following a blink. Knowledge about surface spreading is mostly obtained from studies of the lung where the alveolar lining consists of, an aqueous hypophase covered by a lipoprotein monolayer with dipalmitoyl-phosphatidylcholine as its most important component [326]. I*n vivo,* lipids spread over water or over physiological saline. This movement of the surface lipids and the underlying fluid which is caused by the surface tension gradients is called the Marangoni flow or effect. This spontaneous spreading (or superspreading) ability is not fully understood but factors such as gravity, airflow effects, and surface tension gradients are believed to compete in the spread of thin lipid films. In case of thin film surface layers, surface tension gradients play a more significant role than the other factors and the presence of lipids stabilizes the tear film by:

1) Providing about 25% decrease in the surface tension.
2) Distributing the charge carried by the polar head group
3) The hydroxyl groups of the polar heads interacting with the proteins and mucins in the aqueous subphase [321, 327-329].

As the spreading front stretches, the surface tension (also known as the 'film pressure') increases, even as the concentration of the thin layer reduces. This establishes a dynamic surface tension gradient where the higher the gradient the faster the spreading. This spreading is also dependant upon film thickness, film activity and the viscosity of the underlying fluid. If the film's surface diffusion and gravity are negligible, the unsteady spreading flow generates a wave that travels in the direction of higher surface tension. The film thickens at the edge of the traveling wave and thins behind it, so much so that it may result in a rupture. This rupture causes the spreading to stop. In most of the physiological conditions, a preexisting layer of film may already be present. The leading edge of the newly laid film then spreads more slowly [328]. In the eye, the lipocalins on the inner polar phase

of the lipid layer stabilize the tear film with their surfactant properties [321]. Intact tears have a surface tension of 42-46 mN/m, but when the lipid is extracted the surface tension rises to 53-55.5mN/m. It indicates that the complex of lipocalins and polar lipid fractions of the meibomian with possible contributions from the lacrimal gland origin, maintain the surface tension [288]. The spreading lipid also lowers the surface tension of tears which causes the water to be drawn into the tear film for the aqueous phase of the tear film. Spreading of the lipid film in the eye has been observed *in vitro* by Brewster angle microscopy and in other *in-vivo* studies [320, 330, 331]. These findings suggest that the Marangoni effect probably has a role in maintaining the tear film thickness. In spite of these advances, a recent study on the structure of the surfactant films using the scanning force microscopy revealed that the structure-function relationship of surfactants is more complex and the widely accepted theory of monolayer phospholipids governing the surface tension may probably need further evidence [326].

Compression and expansion of the lipid layer:

The lipid layer of the tear film causes the tear film to act as a thixotropic system (i.e. shear thinning where the bonds and cross-links are labile and can break and reform). This is essential for the fluidization and restructuring of the tear film. It is further suggested that the thixotropic characteristics of the lipid layer are dependant on the interrelationships between the lipid classes and the length of the fatty acids [308]. It is essential for the tear film to maintain its integrity and a reversible compression and subsequent expansion without any hysteresis. *In vitro* studies of lipid monolayers and their formation have shown that either lipid monolayers or bilayers can be formed reversibly. This is dependant on the lipid composition [289]. Using dipalmitoyl-phosphatidylcholine (DPPC), a lipid present in several biological systems including the skin, the tear film of the eye, ear and the alveoli a monolayer to bilayer phase transition was observed and it was concluded that DPPC monolayers can form bilayers spontaneously. Transmission electron microscopy studies reveal that at certain concentrations of DPPC, small folds appeared in the monolayers. These folds represent the over compression of the DPPC monolayer. Studies also indicate that when the composition of the lipid monolayer changes there are differences in the surface pattern appearance. All this lends evidence to the hypothesis that a regular monolayer alone

may not govern the surface tension, but a more complex structure-function relationship exists in the thin layers and it is difficult to conclude that formation of reversible bilayers or trilayers does not occur *in vivo* in the tear film in spite of the lack of evidence [289, 326].

The Aqueous-mucin layer of the tear film:

The mucin component of the aqueous layer:

The mucin part of the aqueous-mucin component of the tear film is formed mainly by the secretion of the goblet cells, and the squamous epithelial cells of the cornea and conjunctiva with a small contribution from the lacrimal gland. The aqueous layer is formed mainly by the secretions of the main and the accessory lacrimal glands. The corneal and conjunctival epithelial cells contribute to the secretion of the aqueous component by secreting water and electrolytes into the tear film [332, 333]. Mucins are high molecular weight glycoproteins with a shape similar to a test tube brush. They contain serine and threonine residues with attached *O*-linked oligosaccharide chains. The carbohydrate chains account for about 70% to 80% of the dry weight of mucins. The molecular mass of mucins range from 3×10^5 to over 4×10^7 kDa [334]. 15 mucin genes have been identified by genome mapping and are partially or completely sequenced (i.e. MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6 to MUC9, MUC11 to MUC13 and MUC16) [335]. Viewed with the atomic force microscope, the ocular mucins are seen as linear polymers with length ranging from several hundred nanometers to several microns and having a diameter less than 1.5nm [336]. Human mucins are categorized into two types: Transmembrane mucins (i.e. a protein subunit in which the polypeptide chain is exposed on both sides of the membrane) and Secretory mucins (which may be gel-forming or the soluble type). In the tear film and the ocular surface epithelium, at least four types of mucins are believed to be present although additional mucins are likely to be present [337]. The mucin concentration gradient decreases from the epithelium towards the lipid layer [286]. The entire ocular surface produces mucins [338-342]. The conjunctival goblet cells synthesize the secretory gel forming mucin MUC5AC (mucin 5, subtypes A and C), whereas the stratified epithelium forms the membrane spanning mucins MUC1 and MUC4 [343].

In humans, rat, mouse and rabbit the goblet cells are seen as plump clusters, and at a high resolution these cells contain packets of mucin. Using fluorescein containing lectins, mucin packets were noted both between the goblet cells and within the goblet cells. Streams of labeled mucin emanating from the apical surface of goblet cells have been visualized [338]. MUC1 is a membrane spanning mucin, expressed by the stratified epithelium of the conjunctiva. MUC1 mRNA has been detected in all layers of the corneal epithelium and in the superficial cells of the cornea, conjunctiva and lacrimal gland tissue [342, 344]. The role of MUC1 is not exactly known though it is believed to facilitate the spread of gel forming mucin. It does not seem to play a significant role in the tear film stability of the MUC1 knockout mice. Although it is not conclusively established, MUC1 may play a role in preventing the adhesion of pathogens to the ocular surface [345-347]. MUC2 is reported in the conjunctival and corneal epithelium and its transcript (a transcript is a sequence of RNA produced by transcription from a DNA template), as determined by quantitative polymerase chain reaction is about 5600 to 6000 fold lower than that reported for MUC5AC [337, 348]. Mucous hyper-secretion is induced both *in vivo* and *in vitro,* by allergic conditions that induce the expression of MUC2 and MUC5AC in the cells [349]. Like the MUC1 the MUC4 is also a membrane spanning mucin reported in all the layers of the conjunctival epithelium, in the limbal portion of the corneal epithelium and the lacrimal gland [286, 337, 338]. The lacrimal gland may therefore be a second source of mucin for the tear film [350]. The conjunctival goblet cells form the gel forming secretory mucin MUC5AC [351]. MUC5B is known to be present in the lacrimal tissue [344, 352]. The conjunctiva and the lacrimal glands also produce the soluble mucin MUC7 [337]. Through the procedure of reverse transcription-polymerase chain reaction (RT-PCR), minor quantities of MUC11 have been detected in the corneal epithelium [353]. The whole of the ocular surface also expresses a highly glycosylated mucoprotein with a high molecular weight. This mucin like glycoprotein complex is recognised by the monoclonal antibody H185 and is expressed at the tip of the microvilli and microplicae of the apical surface of the cornea and the conjunctiva. The antibody recognizes an *O*-linked carbohydrate structure on the mucins [341]. The expression of this mucoprotein is dependant on the density of the microplicae and microvilli of the corneal and conjunctival epithelium. Older cells with less microplicae and microvilli that cause less scattering under the SEM and appear as darker cells have less mucin expression

while the younger cells having more of microvilli and microplicae demonstrate more mucin expression [354]. Normally this shows as a mosaic pattern of binding with the monoclonal antibody H185. In the Dry Eye due to changes in the surface distribution of mucin, the monoclonal antibody H185 binds predominantly to the goblet cell mucin packets, giving the preparation a "starry sky" pattern [355].

Functions of the Ocular Mucins:

Lubrication of the ocular surface:

The mucins on the ocular surface protect the ocular surface and form a physical protective barrier over it. The membrane bound mucins serve as glycocalyx (i.e. a polysaccharide or glycoprotein covering on a cell surface) coating the microvilli and microplicae of the corneal epithelial cells. A chemical attraction exists between the membrane bound mucin complexes and the soluble mucin complexes [286]. During the normal blink the eye lids move rapidly across the eye and this may result in spreading the secreted mucus. The turbulence caused by the blink transfers some of the membrane bound mucin molecules into the aqueous layer [356]. The aqueous mucin component of the tear film lubricates this gliding movement of the lid over the globe. The relative velocity of the gliding surfaces (lid and the globe) is about 15-25 cm/sec with a sheer rate of about 20,000 sec^{-1} and a shear stress of about 150 $dynes/cm^2$ at the mucous-aqueous interface [287]. During this movement the ocular mucins show a non-Newtonian behavior. This means that when a shear force is applied as during blinking, the viscosity of the solution falls. This may be due to the process of shearing and re-entangling of the mucous polymers. In Newtonian behavior the viscosity is independent of the shear rate [357-359]. Both the gel-forming and the transmembrane mucins are important for the spread of the mucin. Another view regarding the ocular surface lubrication and the adhesion of mucin to the epithelium is that the ocular surface cells and the corneal and conjunctival mucous derived from these cells are both hydrophilic. This causes a polar repulsion and prevents the adhesion of the overlying goblet cell mucous to the underlying mucin, to the normal surface cell and the damaged epithelial cells. The ocular mucous therefore remains as a hydrated mucous gel in a sloppy state without adhering to the epithelium. This physical characteristic assists in the spreading of mucous to reduce the shear force of blinking [360].

Stability of the tear film:

Ocular mucins influence the tear-film break up time and play a major role in stabilizing and spreading the tear film [337, 361-363]. The type II cells in the stratified squamous epithelium of the conjunctiva contain numerous vesicles loaded with a long chain mucoprotein. This glycosylated mucoprotein synthesized in the cells is transferred to vesicles which fuse with the outer surface of the conjunctival epithelium. The fusion results in a "mucoprotein spread" that anchors (or repels, according to the polar repulsion concept) and spreads the overlying mucous onto the conjunctival and corneal epithelium [360, 361]. The mucin expressed by the goblet cell is a gel-forming mucin and forms the main component of the mucous layer. It spreads over the glycosylated mucoprotein coating formed by the corneal and the conjunctival epithelium and these two mucins together facilitate the formation of the overlying aqueous layer. In addition to this the mucin in the tears lowers the surface tension aiding in the formation and stability of the tear film. [337]. Other sources of mucin are the stratified epithelium of the conjunctiva which produces the MUC1, MUC4 and MUC7 and the mucins secreted by the lacrimal gland (MUC7) [344]. It is suggested that the membrane spanning mucins spread to provide a negatively charged hydrated epithelial surface which supports and facilitates the distribution of the tear film. When there is a loss of the tear volume along with an altered tear lipid layer, decreased formation of glycocalyx mucins and gel forming mucins, there is an alteration in the mucin gene expression which leads to symptoms of discomfort and dry eye [353]. Mucins therefore play a role in stabilizing the tear film.

Regulation of the mucous layer secretion:

The mucous layer is mainly formed by the goblet cells of the conjunctiva which are single or grouped and connected to the neighbouring cells by tight junctions [364]. These cells in the conjunctival epithelium secrete mucous in an apocrine manner (i.e., all or most of the secretory granules are discharged) upon stimulation. If all the goblet cells of the conjunctiva secrete mucous in response to a stimulus, a rapid depletion of the mucous would occur and there would be no more goblet cell secretion till replenishment occurs. It is therefore possible that there is a neural regulation of the goblet cell. Innervation of the goblet cells by sensory, sympathetic and parasympathetic nerves has been demonstrated suggesting that

goblet cells secrete mucin in response to neural stimulation and a cyclic AMP-dependant signal transduction pathway may be involved [292, 365]. The regulation of the mucin gene expression can be altered by various physiological, pathological and environmental factors such as air pollutants, hormones, bacterial infection, cancer, cystic fibrosis, and embryo implantation. In response to insults from these diverse noxious stimuli a mucin transcription is stimulated probably through common signal transduction cascades [353]. In the respiratory tract airways, it is shown that the epidermal growth factor receptor system (which consists of about 11 members), regulates mucous secretion on being activated by its ligands which are the epidermal growth factor (EGF) and the transforming growth factor α (TGFα). Causes of EGF activation include allergens, cigarette smoke, acute neutrophilic inflammation, bacterial infections and tissue damage [366, 367]. In the tear fluid in humans, both EGF and the TGFα are present and probably originate in the lacrimal gland with a small contribution from the conjunctiva [368-371]. The normal concentration of EGF in minimally stimulated tears is between 0.75-7.1ng/ml [372]. Following reflex tearing there is a decrease in the EGF [373]. In the rabbit the tear EGF concentration rises dramatically after creation of the wound, and returns to the basal level after the 1st post wounding day which indicates its role in epithelial proliferation in the immediate post wound time period [374]. Other chemokines and the interleukins may stimulate the secretion of mucous. Interleukin-9 which is a cytokine produced by the T-helper2 (TH2) cells, specifically stimulates an increased mucin secretion of MUC5AC in the respiratory airway [375]. Mucins therefore play a role in preventing ocular surface inflammation.

The Aqueous component – its formation and function:

The aqueous component of the tear film is mainly secreted by the lacrimal gland and the accessory lacrimal gland. The aqueous tears consist of ions and proteins produced by the secretory epithelium of the glands and the contributions of the plasma cells of the immune system. Its osmolarity ranges from 283 to 304.4 milliOsmols with a slightly higher concentration in the males. An increased osmolarity of aqueous tears (over 310 milliOsmols) is likely to be associated with the condition of Dry Eye [325, 376, 377]. The tear fluid also contains electrolytes such as Na^+ , K^+, Cl^- and HCO_3^- which show an increase by about 3.5% relative to controls in conditions that cause lacrimal gland disease with a resultant Dry

Eye. The protein component of the aqueous tears is derived from the plasma cells and the secretory epithelium of the gland. Plasma cells migrate from lymphoid structures such as the gut associated lymphoid tissue (GALT) and secrete immunoglobulin A (IgA) that protects the ocular surface. Within the lacrimal gland the plasma cells are located in the interstitial spaces between the glands. Acinar cells of the lacrimal gland mainly function to secrete water, synthesize and secrete tear specific proteins and transport IgA from the interstitial compartment into the lumen of the gland. The water is moved from the interstitial spaces of the gland into the lumen of the gland where it is mixed with other secretory products. This movement of water across the epithelium is achieved through the process of osmosis and possibly assisted by two different processes, (a) the aquaporin 5 (AQP5) water channels located in the apical acinar cell and (b) a normal organization of the gap junctions containing the protein connexin 26 and 32 [378]. Aquaporins are water specific membrane channel proteins which enhance water permeability or water plus glycerol permeability (i.e. aquaglyceroporins), in biological membranes. AQP5 is normally expressed at the apical membrane of the lacrimal acinar cells and in the corneal epithelium. In the salivary gland and in the respiratory mucosa it plays an important role in the fluid transport function but its role in the lacrimal gland epithelium is not fully established [379-382]. It is suggested that aquaporins play a role in water transport when a high flow rate of near-isosmolar fluid secretion/absorption is needed. In some patients with Sjogrens's syndrome, an abnormal cytoplasmic localization of the lacrimal APQ5 has been seen [383]. The significance of this finding is unclear as other studies employing different immunohistochemical methods have found that the distribution and the density of aquaporin 5 in the salivary gland is the same in persons with and without primary Sjogren's syndrome [382-384]. The main lacrimal gland specific proteins in the tears are lactoferrin, tear specific prealbumin (TSP or lipocalins) and lysozyme [385]. Protein secretion from the lacrimal gland is stimulated by neurotransmitters and neuropeptides located in the neurons of the glands. The details of the protein secretion are mentioned in the section "Innervation of the Lacrimal Gland". Stimulation of protein secretion causes an intracellular vesicle movement and fusion with the apical membrane of the acinar cell. In addition to this there is a movement of vesicular membrane protein from the basolateral portion of the lacrimal acinar cell into the acinar cell. In the rat the lacrimal tissue secretes the sialomucin complex (SMC)\MUC4 and MUC7. These mucins are a

secondary source of soluble mucin on the aqueous surface. MUC4 is especially important as a deregulation of the SMC\MUC4 expression may suppress apoptosis and facilitate the development of tumors [386].

Morphology of the Lacrimal Gland:

The lacrimal gland consists of lobes of secretory acini that drain into secretory tubules which lead to the main duct of the gland. Acini and secretory tubules are separated by connective tissue, and in some areas they are associated with myoepithelial cells, fibroblasts and mast cells. Scattered throughout the interstitium of the gland are groups of lymphoid cells consisting of B cells, T cells and plasma cells that are scattered in the interstitium of the gland. The acinar cells are columnar while the duct cells are more cuboidal in shape. The apical portion of the acinar and duct cells contains vesicles. A basement membrane upon which the cells lie causes the polarization and functioning of the cell. Large junctional complexes couple the cells in a mechanical and electrical manner at the luminal end of the acini and uncoupling these gap junctions formed by the connexin proteins Cx26 and Cx32, compromises the optimal fluid secretion of the lacrimal gland [378]. Gender and age related changes have been observed in the lacrimal glands including fibrosis of the lobules of the gland, atrophy of the acinar epithelium, and lymphocytic and fatty infiltration of the gland [387, 388].

Innervation and mechanism of secretion of the Lacrimal Gland:

The lacrimal gland is considered to play an important role in the pathophysiology of Dry Eye. Its complex innervation is formed by the sensory nerves, the sympathetic (adrenergic) nerves and the parasympathetic (cholinergic) nerves [389]. The sensory nerves are derived from the trigeminal ganglion, the sympathetic fibers originate in the superior cervical ganglion and the parasympathetic fibers are derived from the pterygopalatine ganglion and the ciliary ganglion [390, 391]. The parasympathetic (cholinergic) nerves contain the neuropeptides VIP (Vasoactive Intestinal Polypeptide), SP (Substance P) and CGRP (Calcitonin gene-related peptide). The lacrimal gland is stimulated by a neural mechanism and by growth factors. The lacrimal nerves stimulate the gland by means of receptors located on the basolateral part of the acinar cell membrane. These receptors include the M_3-receptors (muscarinic-receptors) for acetylcholine, VIP receptors (type I and II), and α_1 and β

adrenergic receptors for norepinephrine. In addition the melanocortin-5-receptor (MC5-R) that responds to the ACTH/α-MSH peptide (adrenocorticotropic hormone/α-melanocyte-stimulating hormone peptide) has been demonstrated in the lacrimal gland. The circulating adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormone (α-MSH) act on this receptor to stimulate protein secretion in the lacrimal gland. [392]. The different regions of the lacrimal gland react differently to the neuropeptides present in the nerves. In the region between the tubules of the lacrimal gland there was a strong reactivity for VIP, while nerve fibers which were associated with the interlobular blood vessels stained for CGRP and NPY [293, 389, 393]. Three different signal transduction pathways activate the lacrimal secretion. These are the parasympathetic muscarinic (i.e. cholinergic) pathway activated by acetylcholine, the α_1–adrenergic agonist pathway activated by norepinephrine and the adenylate cyclase, cyclic adenosine monophosphate (cAMP) dependant pathway that is activated by VIP. The parasympathetic pathway stimulates the muscarinic receptors located on the basolateral membrane of the acinar cells to cause a short term stimulatory regulation of lacrimal gland secretion through acetylcholine (Ach). In the humans only the M_3 muscarinic receptor (glandular subtype) has been identified in the lacrimal gland. The activation of this muscarinic receptor causes it to be coupled to the G-proteins (G_s and $G_{q/11}$, proteins with a high affinity for the guanine nucleotide). The G-proteins are coupled to phospholipase-Cβ (PLCβ). This phospholipase-Cβ acts specifically on a cell membrane constituent, that is involved in signal transduction process (phosphatidylinositol-bisphosphate) and initiates a hydrolysis reaction to produce the second messengers 1,4,5-inositol triphosphate (1,4,5-IP_3) and diacylglycerol (DAG). The inositol triphosphate produced from the hydrolysis reaction interacts with the receptors present on the endoplasmic reticulum of the cell to signal the release of Ca^{2+} into the cytoplasm of the cell. A depletion of the Ca^{2+} stores within the cell can cause an influx of Ca^{2+} across the plasma membrane of the cell. The DAG formed by the hydrolysis reaction causes an activation of the various isoforms of protein kinase C (PKC) present within the cell [394]. These protein kinase C isoforms (*PKC is a family of 11 different isoenzymes*) play a role in modulating the lacrimal secretion. The Ca^{2+} stores released into the acinar cell act either alone or through a Ca^{2+}/calmodulin-dependant kinase pathway and cause the secretion of protein, electrolytes and water from the lacrimal gland. It has been shown that the release of proteins has a direct

relationship to the intracellular acinar Ca^{2+} [395, 396]. While the mechanism of secretion by the muscarinic pathway is well known the α_1–adrenergic pathway is less clear. Stimulation of the sympathetic nerves causes the release of norepinephrine which binds to the α_1- and β-adrenergic receptors on the lacrimal gland. A subtype/subtypes of the α_1- adrenergic receptor which is still to be determined is present in the lacrimal gland and it couples with a subtype of a G protein in the lacrimal acinar cell. This coupling activates an effector enzyme which is as yet unknown. The effector enzyme stimulates specific isoforms of protein kinase C (PKC) which may have an inhibitory or stimulatory effect on the lacrimal gland. The nature of the effect is determined by their localization. The PKC isoforms stimulate secretion by phosphorylating certain protein substrates in the acinar cell, leading to protein and electrolyte secretion. The β-adrenergic agonists are weak stimuli of protein and electrolyte /water secretion [395, 397]. Another signaling pathway in the lacrimal gland is the vasoactive intestinal peptide (VIP) dependant pathway. The vasoactive intestinal peptide interacts with VIP type I and VIP type II receptors (VIPRI and VIPRII respectively), present in the lacrimal gland. While VIPRI has been identified in the acinar and the duct cells VIPRII has been found in the myoepithelial cells surrounding the acini. Upon being activated the VIP receptors couple with a G-protein subtype, to produce adenylate cyclase (AC; formerly called adenylyl cyclase) which in turn produces cyclic adenosine monophosphate (cAMP) from adenosine tri phosphate (ATP). The increased amount of cAMP activates the protein kinase A (PKA), resulting in the phosphorylation of a set of protein substrates which induces through exocytosis a protein and electrolyte/water secretion. VIP also causes an increase in the Ca^{2+} ions. The mechanism for this increase is probably an increase in the influx of extracellular calcium rather than a release of intracellular Ca^{2+}. The VIP pathway is basically a potent stimulator of protein and electrolyte\water secretion [292, 395, 397]. The α-MSH and ACTH both activate the cAMP pathway. α-MSH and ACTH are potent stimulators of protein, but their effect on water and electrolyte is unknown [392]. In addition to these neural mechanisms of stimulation of the lacrimal gland there are different growth factors which cause stimulation of the lacrimal gland. An important family of growth factors identified in the lacrimal gland is the epidermal growth factor family (EGF). The EGF stimulates protein secretion in the rat lacrimal tissue [398]. In the human lacrimal gland the EGF precursor mRNA has been

detected. Soluble EGF has also been detected in the human lacrimal gland. A member of the EGF family called the transforming growth factor α (TGFα) has been detected in the human lacrimal gland. EGF functions by causing the secretion of protein in the lacrimal secretion which affects the function and health of the ocular surface [397]. The lacrimal secretion is inhibited by peptides of the proenkephalin family which are present in the lacrimal gland [399].

Accessory Lacrimal Gland Secretion:

The accessory lacrimal glands are embedded in the conjunctiva. They resemble the lacrimal gland histologically and structurally although true acini are absent and they secrete the same proteins as the lacrimal gland [400, 401]. Each nodule of the accessory lacrimal gland is a functional unit surrounded by a layer of connective tissue. Within the gland there is a ramification of the intralobular ducts and these ducts join to form the main excretory duct. The secretory epithelium of the gland consists of elongated tubules that terminate into end pieces. Within the cells of the tubules are large secretory granules. The amount of granules and organelles in the neighbouring cells vary at a given time lending evidence to neural regulation of the lacrimal gland. Nerve fibers have been demonstrated in accessory lacrimal glands and evidence suggests that in the interstitial connective tissue region of the lacrimal gland, there are blood vessels, fibroblasts and unmyelinated nerve fibers in close apposition to the vascular endothelial cells and the glandular cells. A few axons with parasympathetic and sympathetic characteristics have been identified. All this suggests that there is a neural regulation of the accessory lacrimal gland secretion, with cholinergic agonists stimulating secretion [292, 401, 402].

Secretion by the Corneal Epithelium:

The corneal epithelial cell layer contributes to the formation of the mucinous phase and the aqueous\mucinous phase of the tear film. The epithelium consists mainly of three layers; superficial epithelial cells, wing cells, and basal epithelial cells. These cells upon neural stimulation secrete electrolytes and water into the tears [332, 403, 404]. This secretory process in the corneal epithelium serves to maintain corneal transparency and is partly mediated by multiple ion transporters including the H_2O channels and the Na^+, K^+, Cl^-, H^+-lactate cotransporters (cotransporters are proteins which cause the transport of a substance

across a membrane, and this is coupled with the simultaneous transport of another substance across the same membrane in the same direction). The fluid is transported from the basal to the apical direction and these transporters create osmotic gradients to drive the transport of water. An important ion channel for the passage of water is the Cl^- ion channel where Cl^- secretion is coupled with fluid transport from the stromal to the epithelial side. This fluid transport is achieved by a Na:K pump and cotransporters linked to the Na:K pump such as the $Na^+/K^+/2Cl^-$ cotransporters. In the cornea it is believed that the cotransporter takes up the Cl^- ions from the basolateral membrane and transports it into the cell thus raising the concentration of Cl^- within the cell. This results in an electrical gradient between the stroma and the epithelial cells which causes the opening of the ion channels on the apical side of the epithelium leading to a Cl^- efflux and increased fluid secretion onto the epithelial surface [405-407]. These chloride channel transcripts have been quantified in the human cornea and other epithelial tissue and the Ca^{2+}-activated chloride channel (CLCA2) transcript which is most abundant, of the different functional types of chloride channels in the corneal epithelium may be an important effector of fluid transport [408]. The cornea also maintains hydration by secretion of fluid into tears through the aquaporin channel proteins which enhance water permeability. The two types of aquaporin water channels present on the cornea are aquaporin channel 1 (AQP1) on the endothelial side and the aquaporin channel 5 (AQP5) present on the epithelial side. In the AQP5 null mice a significant increase in the corneal thickness is seen indicating the importance of aquaporins in water transport in the cornea [409]. Apart from the secretion of water and electrolytes the cornea contributes to the tear film by forming the mucoprotein glycocalyx which anchors the overlying mucin and the mucin/aqueous layer of the tear film. The cornea is also known to secrete mucins onto the ocular surface to lubricate the ocular surface and to maintain the stability of the tear film. Mucins secreted by the corneal epithelium include MUC1, MUC2, MUC4 and MUC11 [337, 342, 410]. What is yet not known is the molecular mechanism that couples ion transportation and water and the relative contribution of the various ion channels and aquaporin water channels for the transport of water into the tear film.

Secretion by the Conjunctival Epithelium:

The secretion by the conjunctiva of water and electrolyte is similar to that of the corneal epithelium. The large surface area of the conjunctiva makes it is possible to produce a larger volume of tears when stimulated. The conjunctiva is divided into the palpebral and the bulbar portions and consists of a stratified epithelial layer with an underlying substantia propria. The stratified squamous epithelium is between 2 to 10 cell layers in thickness depending on the region. The limbal epithelium is believed to be about 10 cells deep. On scanning electron microscopy the cells are mostly hexagonal in appearance and are studded with microvilli and microplicae [354, 411]. Five types of cells have been identified on the basis of morphological appearance and the kind of organelles present. These are named as type I cells, type II cells, type III cells, type IV cells and type V cells. The type I cells are the goblet cells of the conjunctiva. These goblet cells have been differentiated from the cell types II-V on the basis of biochemical and immunohistochemical markers [412]. The type II cells contain numerous small granules or vesicles. The type I (goblet cells) and the type II cells play an important role in the protection of the ocular surface.

Innervation of the Conjunctiva:

The conjunctiva is innervated by the sensory, parasympathetic and the sympathetic nerves. They consist of unmyelinated nerves which form a plexus around the base of the epithelial cells and the superficial stroma. The sensory innervation is derived from the first division of the trigeminal nerve [413-415]. These sensory nerves contain the neuropeptides Substance P, calcitonin gene related peptide (CGRP) and gallanin [371, 416]. The parasympathetic nerves are derived from the pterygopalatine ganglion and are supplied via the facial nerve to the conjunctiva. They contain the neurotransmitters, acetylcholine and the vasoactive intestinal polypeptide (VIP) [417]. The sympathetic nerves are derived from the sympathetic plexus of the ophthalmic artery and from the superior cervical ganglion and contain the neurotransmitters, norepinephrine and neuropeptide Y. Sympathetic nerves stimulate the secretion of the stratified squamous cell but not the secretions of the goblet cell [371]. The nerve endings may be free terminals or corpuscular nerve endings. These corpuscular nerve endings are commonly found at the limbus and the margin of the eyelid [418].

Goblet cells of the conjunctiva:

In the humans the goblet cells are found as single cells with an increased density of distribution in the region of the fornices, and in the inferonasal portion of the conjunctiva of the eye. Occasionally clusters of goblet cells may be located within the epithelium as the glands of Manz and crypts of Henle [419]. The mean number of the goblet cells per 100 epithelial cells was about 10.1 ±2.8 in young individuals and was about 5.25 to 3.38 in persons who were 62 years on an average. Others have reported a goblet cell density of 1.24 ± 1.62 in the bulbar conjunctiva compared to 30.21 ± 14.32 in the lower forniceal conjunctiva with no difference due to age and gender [420, 421]. The goblet cells are innervated by the parasympathetic and the sympathetic nerves but not the sensory nerves. Parasympathetic nerves play the main role in stimulating goblet cells and contain acetylcholine and the vasoactive intestinal polypeptide (VIP). They stimulate the muscarinic receptors M1 and M3, while the VIP stimulates the VIPR2 receptors. These receptors are located subjacent to the secretory granules of the goblet cells [422]. The cholinergic agonist signal transduction (i.e. acetylcholine mediated) pathway for stimulation of goblet cell mucous secretion is being elucidated. As in the lacrimal gland and other tissues stimulated by the cholinergic agonists, the second messengers' inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) play an important role in increasing the level of intracellular Ca^{+}. In the rat conjunctiva it is shown that cholinergic agonists bind to the muscarinic receptors to cause a transactivation of the EGFR (epidermal growth factor receptor) that activates mitogen activated protein kinase. This elevates the intracellular Ca^{+} to stimulate goblet cell secretion. The evidence for the role for neural stimulation of goblet cell secretion is therefore increasing [394, 423, 424]. The sympathetic nerves of the conjunctiva and their receptors, the α_{1A}-adrenergic and the β3-adrenergic receptors are present in the region of the basolateral membrane of the goblet cell. The significance of their presence is not clear as it is not confirmed that the adrenergic agonists stimulate goblet cell production [371]. In response to a parasympathetic neural stimulation the goblet cells secrete the gel forming mucin MUC5AC which provides the scaffolding for the mucin layer of the tear film [353, 363]. This mucous secretion is increased when there is an elevation in the Ca^{2+} ions and an activation of the protein kinase C [425, 426]. Other signaling pathways such as the P_2-

purinergic receptors (a class of cell surface receptors which are widely distributed in the central and peripheral nervous system for mediating fast synaptic transmission through ATP) and growth factors such as the epidermal growth factor (EGF) also stimulate the goblet cell mucin secretion [427]. In addition to mucins the goblet cells also synthesize other proteins and fluid. Some of the proteins secreted are peroxidase and the trefoil factor family peptides (TFF-1 and the TFF-3). Their function is unknown and it is suggested that they contribute to the rheological properties of the tear film by mainly increasing the viscosity of the tear film [428, 429]. The goblet cells of the conjunctiva also secrete mucous in response to neural stimulation of the cornea. This may be an immediate paracrine response mechanism to protect the ocular surface [430, 431]. Other sources of conjunctival mucin secretion are the transmembrane mucins and the mucous glycoprotein formed by the second secretory system.

The Second Secretory System:

Apart from the goblet cells which form MUC5AC, the sources of conjunctival mucins are (a) vesicles in the surface cells of the conjunctiva and (b) transmembrane sialomucins (MUC4 and MUC1) secreted by the squamous epithelium. The vesicles of the surface cells (also known as the subsurface vesicles) contain a long chain mucous glycoprotein. These vesicles fuse with the outer surface membrane of the conjunctival cells and expose the intravesicular mucoprotein to the overlying mucous layer. The secreted mucins are distinct from the membrane associated mucins [339, 432]. The stratified squamous cells of the conjunctiva also secrete the transmembrane (i.e. membrane associated) mucins MUC4 and MUC1. These mucins (as the term ‘transmembrane’ suggests) are not released into the tear film but are anchored into the microvilli of the conjunctival epithelial cells by the presence of membrane spanning domains located in the terminal regions of the mucin complexes [353]. All this suggests that neural regulation plays an important role in the production of conjunctival goblet cell secretion in response to physiological, environmental and pathological influences.

Conjunctival fluid secretion and absorption:

The conjunctiva may possibly contribute to the formation of the tear film (including the baseline secretion and reflex secretion) through two main mechanisms. The fluid may be derived from the (a) stratified squamous epithelium of the conjunctiva or from (b) the

innumerous blood vessels present on the conjunctival surface. The epithelial cells form the main source of conjunctival fluid secretion under physiological conditions. Fluid derived from the blood vessels may be due to plasma leaks due to inflammation, antihistamine therapy and conditions that increase the vascular permeability. The fluid flow from the conjunctival surface into the tears is largely due to the active secretion of chloride coupled with fluid flow occurring on the mucosal side of the conjunctiva [433]. The goblet cells also play a role in the secretion and transport of fluid. The conjunctiva is especially important for ocular surface homeostasis, as it performs the functions of, secreting (Cl^- coupled with water) and absorbing (Na^+ coupled with water) fluid at the same time. The fluid secretion is dependant on the rate of Cl^- secretion from the conjunctival mucosa into the tear film and is affected by substances such as calcium ions (Ca^+), adenosine 3′,5′-cyclic monophosphate (cAMP), protein kinase C and uridine triphosphate (UTP) [434-437]. The pathways for the flow of fluid from the serosal side of the conjunctiva to the mucosal side may be either through the tight junctions between the cells (paracellular) or through the cell membranes (transcellular). The transcellular (serosa to mucosa) movement of fluid is the predominant route and this may be aided by the presence of APQ3 (aquaglyceroporin 3) found in the bulbar conjunctival epithelium. Like other aquaporins secretory channels, they may react to the osmotic gradient present across the cell membrane to enhance the flow of water [438, 439]. The exact role of the transconjunctival fluid in ocular surface homeostasis is not clearly defined as the two types of conjunctival secretion are stimulated differently by the parasympathetic and the sympathetic nerves. The formation and the physiology of the tear film is the basis to understand the mechanisms responsible for the stability and break up of the tear film and the resulting sensation.

The Mechanism of tear breakup:

Once the tear film is formed various factors are responsible for the stability of the tear film and its breakup. The stability of the tear film is assessed by different methods such as measurement of the tear breakup time and by measurement of the surface tension of the tear film [440, 441]. The tear breakup time may be measured either by invasive or non-invasive methods. In the invasive method of assessing the tear break up time, the time elapsed from the opening of the eye after a blink to the appearance of the first random dry spot after

application of topical fluorescein to the ocular surface is measured [442]. Non-invasive tear breakup is determined by, the use of grids to study breakup, the tearscope, by methods of interferometry and the Hartmann-Shack wavefront sensor [443-446]. Though many different methods of assessing the tear film exist, the commonly preferred methods include dry eye questionnaires, FBUT, ocular surface staining and Schirmer test [178]. Although the mechanism of the tear breakup is not clearly understood and three different hypotheses of tear breakup have been proposed.

Lipid contamination of the mucous layer:

This hypothesis proposes that an important factor responsible for the spread of the tear film is conjunctival mucin interaction with water and lipids. The formation and rupture of the tear film is explained by the role of the different components of the tear film. Normally the surface of the cornea in the absence of a tear film is hydrophobic. A coating on the hydrophobic corneal surface by bound epithelial mucins renders it hydrophilic and wettable by water [447]. When the eye lid closes it distributes the mucin over the cornea and also eliminates the air-tear film interface. At the lid edges which are in apposition to each other, there is an accumulation of mucous that is coated by the meibomian lipids. Upon eye opening a new tear-air interface of high surface tension is created (70 dynes/cm). As a result the meibomian lipids spread rapidly on this surface. The aqueous phase spreads rapidly under the lipid phase and this is assisted by mucin dissolved in the fluid phase. Due to the lipid and mucin interaction the surface tension of the mucin is lowered (to 35 dynes/cm) and the tear film becomes stable. With the passage of time the tear film deteriorates as the insoluble lipids slowly diffuse towards the mucous-aqueous interface to contaminate the mucous layer. This may cause a rise in the aqueous-mucous interfacial tension and the formation of a hydrophobic region. This hydrophobic locus causes a rupture of the water film resulting in the formation of a hole. This process of lipid diffusion to the mucous membrane could be enhanced by the presence of the Marangoni flow where the movement of the lipids and the underlying aqueous is caused by surface tension gradients. It is not yet determined if the vertical spreading of the meibomian lipids is the primary event that is followed by a spread of the aqueous mucin layer or if the mucin and water layers spread first and subsequent establishment of the lipid layer. Eventually in either case the contamination

of the mucous layer by the tear film lipids results in a reversal of polarity and spontaneous rupture of the tear film [442, 448, 449].

Mucous Rupture resulting in tear film rupture:

According to this hypothesis the mucous layer of the tear film becomes unstable leading to the development of holes and subsequent rupture of the mucous layer leading to the dewetting of the cornea and rupture of the tear film. The normal cornea in the absence of mucous is believed to be hydrophilic and covered by an intense hydrophilic coating of hydrated and relatively insoluble mucous gel while the deeper cells of the cornea are less hydrophilic [450-452]. The mucous layer and the tear film act as a thin biofilm and they are governed by forces such as Van der Waals forces (also termed as apolar forces, Lifshitz forces, London forces or dispersive forces), ionic bonds, hydrogen bonds and acid base interactions (i.e. polar forces) [453]. Van der Walls forces are intermolecular forces which arise from non-specific attractions when two molecules or atoms are close together. These forces act on all atoms and molecules including ones that are neutral. These forces can act over long distances and also cause an orientation of the molecules. Polar (acid/Base) forces act over a short range and the force of attraction is inversely related to the distance. Apolar or Lifshitz forces normally favor adhesion (although these forces are attractive, they can also be repulsive in nature), while the polar forces cause repulsion. The polar repulsive properties of the normal uncontaminated mucous prevents the cohesion of the mucous to itself and its adhesion to the underlying epithelium [454]. It is suggested that the mucous present in the tear film therefore acts as a "sloppy gel" which cannot adhere tightly to the epithelium. The mucous layer formed by the conjunctival and lacrimal secretion is believed to be separated from the mucoprotein glycocalyx by a potential distance of about 5nm containing an electrolyte-water mix. Due to factors such as dehydration, epithelial damage, cell loss and lipid contamination the mucous looses its polar properties resulting in a binding of mucous to the epithelium. This collapse of the hydrophilic mucous may result in an immediate breakup of the tear film on the surface. In the *in vitro* experiments it is shown that viscous polymer films of high interfacial tension (about 40mJ m^{-2}) spontaneously dewet in a few minutes when they are present in between water and a substrate and when the effective Hamaker constant is positive (the Hamaker constant is a constant governing the strength of

the Van der Walls forces, which cause dewetting) [455]. In addition to this spontaneous dewetting when the interfacial tension is low as in the case of the mucous-aqueous interfacial tension the breakup of the tear film occurs in a few minutes. These observations propose that under the influence of the Van der Walls dispersion forces the mucous layer may rupture to cause a spontaneous dewetting of the cornea and this phenomenon is exacerbated in dry eyes. Once the rupture of the mucous layer occurs, the aqueous tears come directly into contact with the corneal epithelium. However because the epithelium is hydrophilic, tear breakup is initiated when the tears come in contact with hydrophobic epithelial sites which are non-wettable by the aqueous tears.

The above two hypotheses both suggest that the surface of the cornea changes from a hydrophilic to a hydrophobic state. A different model proposes that tear film thinning is based on the upper lid velocity during the blink phase.

The hydrodynamic coating model:

This hypothesis proposes that the thickness of the tear film is a function of the velocity of the upper lid's movement. According to this coating model, as the upper lid rises up a fluid surface layer is first created along the rising meniscus of the upper lid. This rise is determined by the velocity of the upper lid movement and the radius of curvature of the tear film meniscus at the lid margin. The fluid rise is followed by the slower rise of the thicker lipid layer. The rise of the lipid layer may cause a reforming of the tear film and any thickness disturbance to the bulk of the tear film is evened out by a curvature driven leveling with the intermolecular forces acting on the thin film. The deposition of the tear film is therefore governed by two opposing forces, the viscosity of the tears which resists the upward drag of the rising tear film, and the capillary force and surface tension which draw liquid into the tear film. When the upper lid stops moving, the drag force diminishes while the tear film continues to draw fluid resulting in a thinning of the tear film close to the lid margins. In studies employing fluorescein this may be observed as black lines adjacent to the upper and the lower lid. The tear film is thus perched between the upper and lower lid margin. The formation of the black lines depends on the initial tear film thickness (which is a function of the velocity of the upper lid's movement) and a thinning dependent on the radius of curvature of the tear menisci at the margins of the lids. The exact mechanism of

tear breakup is not clearly explained though is proposed that either drainage due to gravity or a rising film height reaching the effective range of the dewetting forces may be responsible for the breakup of the tear film [313, 456].

In all the three cases a breakup of the tear film is proposed. Studies of the tear film have indicated that the depth of the tear breakup may be up to 1.5μm thick.

With this brief account of the formation and function of the tear film, the physiology of the cornea and its role in ocular discomfort will be considered.

6. Corneal nerves - structure and functions:

The structure and function of the cornea has intrigued researchers for more than two centuries. Early studies about the cornea were documented in the eighteenth century, by the anatomist Antoine Pierre Demours (1762-1836). Demours Sr., father of Antoine Pierre Demours, published numerous essays on ocular anatomy and is often credited with the first description of the 'posterior membrane' of the cornea now known as Descemet's membrane. [457]. The corneal innervation in mammals was probably described about one hundred and sixty years ago [458]. In spite of many different studies over this period, many questions regarding the architecture and physiology of the corneal nerves remain unanswered. A brief description of the physiology and function of the corneal nerves and factors affecting corneal sensations follows.

The innervation of the cornea – anatomy and architecture:

The cornea is supplied by the sensory, sympathetic and parasympathetic systems and is among the densely innervated structures in the body with the number of nociceptors estimated at about 7000 per mm^2 [459].

The sensory nerve supply to the cornea:

Embryology and main divisions of the trigeminal nerve:

Retrograde nerve tracing studies mostly done in animals indicate that the sensory nerves of the cornea originate mainly in the ophthalmic division of the ipsilateral trigeminal ganglion. The cells of the trigeminal ganglion have central processes that form the large sensory root of the fifth cranial nerve while the peripheral processes separate into the ophthalmic, maxillary and mandibular divisions of the trigeminal nerve. Embryologically the trigeminal nerve is the nerve of the first branchial arch although in humans the ophthalmic branch of the trigeminal is not a branchial component and therefore does not supply any of the structures formed by the pharyngeal arches. Histological studies of the Gasserian ganglion's development in the embryo indicate that the cells of the ophthalmic nerve are separate from the maxillary and mandibular nerves which are more close together [460, 461]. Each division of the trigeminal nerve is associated with an autonomic component. The ophthalmic

division is associated with the ciliary ganglion, the maxillary division is associated with the sphenopalatine ganglion and the mandibular division is associated with the otic ganglion. The sympathetic innervation to the cornea is derived from the superior cervical ganglion. Parasympathetic innervation from the ciliary ganglion is present in the rat and the cat corneas, while in humans the parasympathetic innervation is unclear. The ophthalmic nerve is the smallest of the three branches of the trigeminal and contains most of the corneal afferent nerves. These branches reach the eye mainly via the long and short ciliary nerves, which arise from the naso-ciliary branch of the ophthalmic nerve. These afferent sensory nerves are small in size and their properties of conduction are similar to nerves in the range of the C-fiber and A-δ fibers. After piercing the sclera the autonomic and the sensory fibers course toward the anterior segment in the suprachoroidal space between the choroid and the sclera. In the suprachoroidal space they branch to exchange axons so that at the corneoscleral limbus each bundle contains sensory, sympathetic and parasympathetic nerves. As the nerves approach the cornea they move anteriorly to separate from those supplying the uvea. Before entering the stroma the majority of fibers give branches to the limbal blood vessels and proceeds further as corneal nerves.

The morphology and architecture of the corneal afferent nerves:

The corneal nerves enter the cornea at about the anterior third of the stroma and the nerves adjacent to each other branch to rejoin and form a plexiform network that is distributed in a radial fashion at the periphery of the cornea. In the humans the corneal nerves form about 70-80 fascicles or bundles with each fascicle containing about 900 to 1500 axons [458]. Upon entering the cornea they loose their perineurium and myelin within a region of 1mm of the limbus. These nerves are enmeshed in an extracellular matrix and lie close to keratocytes which are often wrapped around the nerve bundle. Whether some of the sensory nerves end in the stroma is not completely determined. The majority of the stromal nerves form a subepithelial plexus beneath the basement membrane and then turn up at 90 degrees and proceed towards the corneal surface by penetrating the Bowman's layer throughout the periphery and the centre of the cornea [462, 463]. After piercing the basement membrane, the large nerve bundles turn 90 degrees again and proceed in a direction parallel to the surface of the cornea between the Bowman's layer and the basal epithelial cell layer as

epithelial leashes [464]. Another commonly observed morphology is straight nerve terminals originating directly from the sub-epithelial plexus. These nerve terminals pierce the Bowman' membrane and ascend in the epithelium. They may undergo a variable amount of branching before terminating in the superficial layers of the epithelium just a few microns beneath the corneal surface [462, 465, 466]. The basal epithelial leashes contain both beaded and straight nerve fibers and the straight nerve terminals. From the basal leashes only the beaded nerve fibers bifurcate and turn up, towards the anterior surface of the epithelium to end as axon terminals in the superficial epithelium. The size of the nerve fibers in the subbasal plexus is commonly between 0.1μm to 0.5μm although they may range between 0.05μm to 2.5μm. These subbasal leashes of nerves are unique to the cornea. They approach the apex of the cornea radially along the 2-8, 3-9, 4-10, 5-11 and 6-12 directions. Except for leashes in the 6-12 direction, the other leashes do not reach the corneal apex nor do they cross to the other side of the cornea. Only the leashes along the 6-12 hr direction which have a superior-inferior orientation reach the apex of the cornea. These observations suggest that in the region of the corneal apex the preferred orientation of the nerves is in the superior-inferior direction, while in the region surrounding the apex the nerves are in the nasal-temporal direction [459]. Each leash consists of 2-15 tightly packed thin axons. The individual axons traverse the cornea and sometimes branch dichotomously. They occasionally interconnect by cross bridges with the adjacent axons, before finally ending as free nerve terminals. In some cases the nerves may travel for as long as 2 millimeters in the human eye before ending as free terminals [467]. As a result of this long path of the nerve fibers the receptive field of a single sensory axon may cover a region from 20% – 50% of the corneal surface [458, 468].

Studies have been done to understand the architecture of the nerves in relation to the electrophysiology and the function of the nerves. In the rabbit the Aδ and C fibers of the cornea show different distribution patterns although such differences are not clear in humans [469]. These studies using the methods of light microscopic examination, electron microscopic examination mainly revealed the density and ultrastructure of the corneal nerves.

Histology and Density of the corneal nerves:

Light microscopic, electron microscopic and confocal microscopic appearance of the nerves reveal that the nerves with a beaded appearance invaginate the epithelial cells and keratocytes, suggesting a direct innervation of the cells [463, 470]. In the stroma the nerves contain vesicles and mitochondria that are homogenously distributed. In the epithelium the varicosities on the beaded nerve fibers mainly contain clear vesicles and mitochondria and at the location of the beads the nerve fibers turn upwards. In the upward course the nerves run between the epithelial cells and may also deeply invaginate into the cells. At the level of the wing cells they are swollen and contain large dense vesicles. Different studies have quantified the neural density using different methods [464, 470]. It is suggested that the density of the nerves may be the same in the central and the central-peripheral portion of the cornea. The overall neural density (defined as the total length of the nerve fibers within a defined optical section frame, of the confocal microscope), as observed during in vivo confocal microscopy in the epithelium of younger individuals (25 ± 5 years of age) was about 632.35 ± 287.57 $\mu m/mm^2$ and in the older individuals (70 ± 5 years of age) was about 582.39 ± 327.13 $\mu m/mm^2$ [471].

The autonomic nerve supply to the cornea:

The function of the autonomic system in the human cornea is not completely understood. In animal studies the cornea receives a modest amount of innervation from the superior cervical ganglion [472]. In humans it is believed that the post-ganglionic sympathetic fibers exit from the superior cervical ganglion in the internal carotid nerve and ascend with the internal carotid plexus. These fibers then enter the carotid canal in the petrous portion of the temporal bone and at the foramen lacerum the sympathetic fibers destined to reach the eye, move away from the artery to advance towards the trigeminal ganglion. Before entering the orbit the sympathetic nerve fibers, form a plexus with the parasympathetic fibers derived from the pterygopalatine ganglion. This plexus forms a meshwork along the abducens, the trochlear and the ophthalmic nerves. The further course of the plexus along the ophthalmic nerve has been inferred from clinical observations in the humans and by retrograde tracing studies or selective denervations done in animals. Most sympathetic nerves pass to the naso-ciliary nerve and then to the long and the short ciliary nerves to reach the anterior segment of

the eye [473, 474]. The sympathetic innervation of the cornea is largely derived from nerve fibers present in the corneoscleral limbal region. A stromal penetration of the nerve fibers has been observed and in some instances an intraepithelial penetration of the cornea is also noted [465, 475-477]. In humans, the anatomy of the parasympathetic system supplying the cornea is not clear although recent findings indicate that a dysfunction of the parasympathetic system may contribute to the condition of Primary Sjogren's Syndrome [478]. The sensory and autonomic nerves of the cornea exert their influence on the epithelial cells and respond to the challenges of the environment by expressing different neurochemicals which are discussed below.

Ultrastructure and histochemistry of the corneal nerves:

Based on anatomy, function, histochemistry, and dependence on trophic factors the corneal nerves, like nociceptors in other parts of the body, can be divided into two broad populations of nerves: Neurons with myelinated medium sized (2-6 μm) axons which have a fast (12-30 m sec^{-1}) conduction and large diameter cell bodies (Aδ-fibers) and unmyelinated, thin (0.4–1.2 μm) slow (0.5-2.0 m sec^{-1}) conducting fibers with a cell body of small diameter (C-fibers). Based on the anatomical ultrastructure three main types of nerve endings are believed to exist. The first type contains numerous mitochondria, neurofilaments and microtubules with occasional small and clear round vesicles. The second type of nerve fiber contains many small and clear vesicles with occasional large or small dense cored vesicles. The dense cored vesicles contain neuropeptides such as Substance-P and CGRP (calcitonin gene related peptide) but the contents of the clear vesicles is not known as yet. In animal studies these clear vesicles were present even after a combined autonomic ganglionectomy though they disappeared after a sensory denervation [479, 480]. The third type of nerve contains numerous small dense cored vesicles. These vesicles disappear after superior cervical ganglionectomy and therefore are believed to be derived from the corneal sympathetic nerve supply. Recently it was proposed that the nerves of the cornea are homogenous in appearance and that the differences in the morphology previously reported were due to differences in the segment of the nerve terminal that was cut [463]. Through the techniques of histochemistry different receptor antibodies have indicated the presence of various types of sensory receptors especially for the transduction of the noxious stimuli.

Although there are few studies directly indicating the presence of receptors on the corneal nerves, there is indirect evidence from animal studies and from studies involving the detection of noxious stimuli that point to the presence of receptors on peripheral terminal endings such as the corneal nerves. A few of the important receptor types present on the cornea include:

Ligand-Gated Ion Channels:

The ligand gated ion channels are grouped into three structurally distinct families which are not phylogenetically related and may be either excitatory or inhibitory. These families are:

1) **Cysteine-loop (Cys-loop) receptor family:**

   This includes the cys-loop receptors made of 5 homologous subunits of proteins which traverse the cell membrane four times (i.e. they have 4 transmembrane domains). The anionic Cys-loop receptors include the GABAA and the glycine receptors and the cationic receptors include the nicotinic and 5-HT3 receptor. In the rat, the application of GABA activators on the cornea has indicated that GABAA receptor mechanisms modify corneal input to the second order neurons in the trigeminal brainstem complex [481].

2) **Glutamate receptors:**

   Glutamate is a major excitatory amino acid transmitter that acts on the NMDA (N-methyl d-aspartate) and the non NMDA ionotropic glutamate receptors (ionotropic receptors are the ligand gated ion channels). The glutmate receptors have four homologous subunits of proteins (i.e. three transmembrane domains) and in the rat these receptors localized on the cornea are involved in transmitting the excitatory amino acid to the central trigeminal neurons to cause an increase in the c-fos expression when there is a stimulation of the cornea [482].

3) **ATP gated ion channel:**

   The application of ATP opens the ionotropic channel known as the P2X receptors and the metabotropic G-protein coupled receptor termed as the P2Y receptors [483]. Of the seven subtypes of the P2X receptors the commonly found subtypes on the nociceptors are the $P2X_3$ and the $P2X_2$ subtypes. The ATP-gated cation channels have two transmembrane domains and bear a structural similarity to the mechanosensitive

channels. The purines adenosine triphosphatase and adenosine when applied peripherally have nociceptive as well as antinociceptive properties. In the rabbit cornea and the rat cornea the application of ATP elicits an increase in the intracellular Ca2+ ion concentration indicating the presence of receptors sensitive to ATP [484]. Recently using the rat model to study sensitization and hypersensitivity to pain it was shown that a $P2X_4$ receptor subtype located on the microglial cells was a key pain receptor [485].

Excitatory Amino Acids:

The excitatory amino acids (EAAs) such as glutamate and aspartate act as metabotropic receptors and are coupled to intracellular membrane-associated proteins termed as G-proteins which serve as the second messengers or transducers of the nociceptor initiated response (G-proteins are activated by several receptors and normally have a high affinity for guanine nucleotides and hence are named as G-proteins). The EAAs may also act as ionotropic receptors when they are coupled directly to cation permeable ion channels. Although there is no definite proof as yet, it is believed that the corneal nerves may be activated by these EAAs [459].

Substance P and CGRP receptors:

Substance P is a neuropeptide which activates the neurokinin-1 receptors. In humans Substance P is seen in the beaded fibers of the corneal epithelium and in the nerve trunks of the corneal stroma. These nerves originate in peptidergic neurons located in the trigeminal ganglion and disappear after maxillary and ophthalmic neurotomy [486, 487]. The CGRP co-localizes with Substance P and is demonstrated in the nerve fibers of the cornea and the limbal blood vessels [488].

Vanilloid Receptors:

The vanilloid receptors belong to the TRP family of channel proteins. The vanilloid receptors (VR1) are expressed on nociceptors and they bind capsaicin. The receptors are transducers of noxious thermal and chemical stimuli. In rats VR1-immunoreactivity was determined in the small diameter nerve fibers of the cornea and the application of the agonist capsaicin caused a transient increase in the intracellular $Ca2^+$ ion concentration in the nerve terminals which was blocked by the capsaicin antagonists [484, 489].

Tetrodotoxin sensitive and Tetrodotoxin resistant Na+ channels:

The marine neurotoxin Tetrodotoxin selectively blocks the voltage sensitive ion channels by inhibiting the inward movement of sodium with no effect on the movement of potassium leading to an inhibition in nerve conduction. The corneal nerves express different types of voltage-gated Na(+) channels which are either sensitive or resistant to the effects of Tetrodotoxin. At least two types of TTX resistant sodium channels are present along the entire length of the peripheral corneal nerve fiber from the region of the corneoscleral region to the distal end of the corneal leash fiber[490]. In the trigeminal ganglion corneal and non-corneal nerves responded differently to Tetrodotoxin. The fast conduction nerve fibers i.e. the myelinated A with > 1.5m/s were sensitive to Tetrodotoxin and the slow conduction nerve fibers i.e. the unmyelinated C with < or = 1.5m/s were resistant to the effect of Tetrodotoxin indicating that the terminal characteristics of the nerve endings play an important role in determining the properties of nerves [491].

Serotonin Receptors (5HT):

Serotonin is an algesic and inflammatory mediator. Corneal sympathetic nerves are believed to contain serotonin and it is suggested that this amine has a role in the corneal nerves [492, 493]. The activity of neurons in the rat trigeminal subnucleus was studied by the application of 5HT to specific regions of the cornea. 5HT evoked little response compared to other irritant chemicals. This could be due to very little or no central transmission of 5HT receptors [494, 495].

Opioid Receptors:

The opiod growth factor (OGF) and its receptors termed as “OGFr” are widely present in the corneal epithelium of different species such as the rat, cat, dog, horse and man. In humans OGF and OGFr have been localized in the epithelial cells of the cornea [496]. The OGF functions to inhibit would healing in a receptor mediated fashion and in the rat small quantities of OGF are expressed in the corneal nerve fibers [497, 498].

Prostaglandin Receptors:

Prostaglandins are local mediators of inflammation and they regulate the cell function by acting on the G-protein coupled cell surface receptors. There is no anatomic data which

documents the presence of prostaglandin receptors on the nociceptors on the ocular surface. The actions of prostaglandins such as PGE_2 are mediated by specific E-prostanoid receptors (EP) of which there are at least four subtypes (EP_1 through EP_4) and the actions of $PGF_{2\alpha}$ are mediated by the FP receptors. All four types of EP receptors have been found in the conjunctiva and the cornea. The levels of EP_1 receptor subtype and the FP receptor protein were especially high in the conjunctiva and cornea [499].

Acetylcholine receptors:

The application of acetylcholine activates the corneal nociceptors in a dose dependant manner [500]. Acetylcholine normally causes a burning sensation upon application and both the cholinergic and the muscarinic receptors are involved in this process. In the rabbit cornea acetylcholine activated a specific population of nerve fibers not activated by mechanical or thermal stimuli and physiologically these nerves were involved in the transmission of pain following injury or ischemia [501].

The neurochemistry of the corneal nerves:

Based on neurochemistry, corneal nerves may be peptidergic or non-peptidergic. Peptidergic nerves include the sensory and autonomic nerves of the cornea that express neuropeptides [463, 465]. The neuropeptides act on the receptors located on the terminal afferent corneal fibers and initiate nociception and somatic sensation. At least 17 different neuropeptides of neuronal origin act as ligands to stimulate the corneal nerves. Apart from these neuronal neuropeptides the corneal peripheral nerves are also under the influence of non-neuronal ligands such as Acetylcholine, ATP, prostaglandin E, opioids, adenosine, glutamate, bradykinin, noradrenaline and serotonin. The peptidergic sensory nerves of the cornea express tachykinins which include Substance P and neurokinin A, Calcitonin CGRP, Galanin, Pituitary adenylate cyclase-activating peptide (PACAP) and the Vasoactive intestinal polypeptide (VIP). The different ligands present in the cornea and the various types of receptors indicate that different receptors may respond to different ligands and the sensory input may possibly be modulated before it is transmitted centrally. Sensitization is an example of peripheral modulation of the nociceptors where there is a decrease in the threshold and an increased sensitivity of the nociceptors to heat and chemical suprathreshold stimuli [502, 503]. Inflammatory mediators such as histamine, prostaglandins, bradykinin,

and 5HT all sensitize the peripheral nociceptors and this is demonstrated in the polymodal nociceptors in the cornea [504, 505]. All this indicates that following a nociceptive stimulus it is possible that different nociceptors are activated peripherally and the evoked response to a stimulus is due to the co-activation of various receptors. The nociceptors may therefore modulate the response to a noxious stimulus at the peripheral level. The stimulation of the nerves causes an orthodromic propagation of impulses to the CNS and an anitdromic impulse may trigger the release of neuropeptides from the nerve terminals into the extracellular space. These ligands diffuse to specific receptors on distant nerve terminals and this process termed 'volume transmission' is an important method for neurotransmission in the peripheral nervous system. When the receptors are activated by ligands released from the same terminal the process is termed as autoreception. Another method of modulation of the peripheral sensory terminal is by paracrine reception where the ligand is released from one terminal and diffuses to a neighboring terminal to activate the receptors of the terminal. Considering that there are innumerable neuropeptides in the nerves of the cornea and in the different components of the 'lacrimal gland/ocular surface functional unit' it is possible that the methods of volume transmission, autoreception and paracrine reception are all methods initiating the sensation of discomfort and pain in the anterior ocular surface. The peptidergic nerves containing Substance P and CGRP probably originate in the trigeminal ganglion and are dependant upon the Nerve growth factor (NGF) neurotrophin for development. They are seen to disappear after ophthalmic and maxillary neurotomy. The non-peptidergic nerves in the cornea do not contain any of these peptides but express a sensory neuron specific acid phosphatase isoenzyme. This substance termed as fluoride-resistant acid phosphatase (FRAP) is believed to use the excitatory amino acid neurotransmitters aspartate and glutamate. The non-peptidergic nerves are dependant on the glial cell derived nerve growth factor neurotrophin (GDNF) for development. Normally the corneal nerves and the corneal epithelial cells support and exert a trophic influence on each other. As a part of the tissue maintenance and physiological renewal, the neuropeptides of the trigeminal and sympathetic neurons stimulate the corneal epithelial cells and modulate the proliferation of the corneal epithelium. Substance P causes an increase in the corneal epithelial cell mitotic activity and CGRP causing inhibition of epithelial cell mitosis [506]. These trophic factors are normally released into tears and during "resting conditions" they prevent corneal damage caused by

minor insults such as mechanical movement of the eyelid, blinking, desiccation, changes in humidity, cooling and air currents [459]. The cells of the corneal epithelium support the nerves by releasing growth factors essential for the nerve such as Nerve Growth Factor (NGF) and the Gial cell derived neurotrophic factor (GDNF). The NGF and the GDNF regulate the sensitivity of the nociceptors and also act as inflammatory mediators [507]. It is now known that inflammation and nerve injury result in a phenotypic alteration of the neuropeptides and this has a functional significance during the inflammatory process [508].

The neuropeptides of the autonomic nerves of the cornea:

The sympathetic autonomic nerves of the cornea contain the neurotransmitters, noradrenaline, serotonin and neuropeptide Y (NPY). While the parasympathetic nerves are not described in the human cornea, in animal studies involving the rat the neuropeptides such as acetylcholine, VIP, met-enkephalin, NPY, and galanin have been described. Some of the other neuropeptides detected include cholecystokinin, brain natriuretic peptide, vasopressin, neurotensin and beta endorphin. The origin of these neuropeptides is not yet clear and it remains to be determined if they are of sensory or autonomic in origin.

The functional characteristics of the sensory corneal nerves:

General characteristics:

The corneal sensory nerves are nociceptors and like other nociceptors possess the following characteristics.

1) **Lack of Specificity:**

   Sensory nerves which detect odor, light or tactile stimulus often exhibit specificity to a stimulus modality. The olfactory receptors are very specific and each receptor type responds in a specific manner to a specific odor from among more than a thousand different types of odor. The nociceptors differ from these sensory nerves as they lack specificity and most of them are polymodal and respond to mechanical stimuli, noxious heat, cold and endogenous and exogenous chemical stimuli. These differing characteristics evolved in nature for the survival of the animal [509, 510].

2) **Low degree of gain:**

In the senses concerning vision and odor the energy of a low intensity stimulus is amplified so that a high gain converts the stimulus into an electrical energy that can be detected. In the nociceptors a low intensity stimulus from an external source does not translate into high gain as low intensity stimuli often do not cause tissue damage. Any stimulus that might cause damage to the tissue often does not need amplification to be understood. The exception to this mechanism is endogenous stimuli caused by cytokines and substances liberated as inflammatory products which are detected with a high sensitivity [510].

3) **Adaptation and Sensitization:**

When a constant mild stimulus that does not result in tissue damage is present, then the response to the stimulus is often reduced in about 2-3 seconds to prevent a saturation of the response. This is the process of adaptation. However if there is any preceding tissue damage, even a non-nociceptive input from an undamaged region near to the site of the injury produces a sensation of discomfort or pain. This is the process of sensitization and it is believed to be an intrinsic property of the nociceptive terminals resulting in a state of hyperalgesia. The inflammatory mediators and calcium ions are especially involved in the process of sensitization.

4) **Fatigue:**

This is the property of the nociceptors where following adaptation there is a slow recovery and often the response may be after a certain length of time. Fatigue has been demonstrated in polymodal nociceptors innervating the cornea [502, 511].

These properties influence the mechanisms involved in the transduction and propagation of sensations that give rise to discomfort and pain. The steps involved in transducing different forms of energy in nociceptors are yet to be fully understood. Also very little information is available about the modulation and onward transmission of the stimulus by structures surrounding the corneal nerve terminals. Based on the response to the different types of energies, it can be presumed that the corneal and conjunctival nociceptors have different transducing mechanisms for each form of stimulating energy and on this basis, the ocular

nociceptors are classified as mechanosensitive nociceptors, mechanoheat nociceptors, polymodal nociceptors, cold nociceptors or mechano-insensitive nociceptors.

Classification of Neurons based on Electrophysiological response:

The mechano-sensory neurons:

This is a class of nerves that respond exclusively to mechanical forces. These nerves have the highest conduction velocity and action potentials with large amplitude [511, 512]. They are more responsive to a moving stimulus rather than to a sustained indentation. The impulse response of these mechanosensory nerves in terms of duration, latency and frequency of their action potentials is proportional to the amplitude and velocity of the stimulus. A sustained indentation normally causes an adaptation in the response [513]. By employing mechanical and electrical stimuli upon small spots of the cornea the receptive field of the mechanosensory units was plotted as having an elongated shape along the trajectory of the nerve fiber. Stimuli which moved parallel to the long axis of the nerve fiber produced a maximal stimulation while stimuli applied perpendicularly had a diminished response [469]. The mechanical thresholds of the human cornea as measured by the Gas esthesiometer as a response to a puff of air is 82.8 ± 13.8 mL/min while others reported similar corneal threshold values in the range of 80 ± 6 mL/min. The conjunctival threshold values in these studies were 84.9 ± 10.4 mL/min and 140±10 mL/min respectively. The sensations evoked by mechanical stimulation were described as unpleasant irritation, burning and stinging [264, 514, 515]. In the cat, the bulbar conjunctiva surrounding the cornea contains low threshold mechanosensitive receptors [516]. In the human eye similar corpuscular nerve endings are noted in the region of the limbus though their function is not fully determined [418]. The limbus and the adjacent bulbar conjunctival region is innervated by the conjunctival and scleral units and also from collaterals of corneal axons termed the sclero-corneal units. The receptive fields of the scleral units are more abundant in the anterior region of the eye although they extend over the whole eye. The receptive fields of the sclero-corneal units extend from the cornea to the bulbar and sometimes palpebral conjunctiva. Mechanical threshold of these units is low in the limbus and high in the region of the conjunctiva and their response to mechanical force and heat closely resembles the responses of corneal mechanosensitive and polymodal units [516].

The mechano-heat neurons:

The detection of heat is subserved by specialized channels termed as the transient receptor potential vanniloid-class of channels (TRPV) [517]. Four types of TRPV channels are implicated in sensing heat [518]. In the cornea of the cat are present a group of Aδ neurons with mechanical and thermal sensitivity but no chemical sensitivity except when stimulated by repeated applications. Similarly in the rabbit cornea the Aδ units respond to high intensity mechanical stimulus (> 350 dyne) and a high heat stimulus (> 40^{o} C) with a bimodal phasic pattern but do not respond to ACh [466]. Although less in number compared to the polymodal neurons in the cat cornea, the mechanoreceptors constitute about 22% of Aδ fibers and 15% of mechano-heat receptors. Threshold with thermal stimulation of the cornea and conjunctiva was in the range of 1.4 ± 0.1^{o}C and 2.0 ± 0.1^{o} C [514].

Cold Neurons:

The transduction of cold into electrical activity is now believed to be due to cold receptors present on the nerve terminals. Receptors sensing cold have been cloned and in the rat trigeminal ganglion a member of the transient receptor protein channel responding to cold stimuli has been cloned [519-521]. In cultured dorsal root ganglion neurons the cold receptor stimulant menthol and cooling initiated ionic currents and like other nociceptors these neurons demonstrated the properties of sensitization to menthol, adaptation to sustained cooling and modulation by calcium [522]. Cold units in the cat cornea respond to drop in temperature vigorously and the frequency of these impulse discharges are proportional to the drop of temperature. These cold neurons have small receptive fields ($4mm^2$) located in the periphery of the cornea. The corneal cold units are functionally different from the conjunctival and scleral cold units as they have a weak response to mechanical and chemical stimuli unlike conjunctival and scleral units which are insensitive [516]. There are also functional differences existing between cold sensitive nerves and polymodal nerves in the guinea pig cornea. It is suggested that the structural differences at the nerve terminals may actively propagate sensory action potentials from polymodal nerves while in the cold sensitive nerves that have fewer Na^{+} channels there is a passive initiation of the action potential at a more proximal point in the axon [523]. Cold air applied to the human cornea elicits an initial cooling response and the cooling threshold is established as -2.4 ± 0.4^{o} C

below the corneal surface temperature. Others investigators have reported that a 0.3^{o}C drop in tear film surface temperature caused a cooling effect [524]. The initial cooling is followed by irritation as the temperature is further reduced to about -5^{o}C below the corneal surface temperature. Menthol applied to the eye causes an initial cooling sensation followed by mild irritation or discomfort and in some cases caused a sensation of burning possibly due to simultaneous activation of cold and polymodal neurons [264, 524].

The polymodal neurons:

These polymodal neurons have an abundant distribution (71% in the cat cornea), the ability to detect a wide range of stimuli and are also easily sensitized by mediators such as heat and prostaglandins [511, 513, 525]. Functionally they are Aδ and C neurons which respond to mechanical, thermal and chemical stimuli.

Response to mechanical forces:

As a response to mechanical force the electrical recordings of polymodal units show a spontaneous activity with lower mechanical threshold than pure mechanosensory nerves, a tonic discharge to sustained mechanical indentation, a long lasting postdischarge and fatigue to repeated stimuli of high intensity [502].

Heat responses:

The psychophysical perception of temperature is due to specific classes of neurons that respond to different ranges of temperatures. The polymodal neurons respond to heat above 38-39^{o}C. Most of the capsaicin receptors encode noxious heat and are activated by low to moderate heat at about 43^{o} C to a sudden suprathreshold temperature elevation. The nerves respond with impulses that accelerate in frequency and subsequently peak. A gradual temperature increase to a noxious level causes a proportional increase in the firing frequency. If the temperature exceeds the noxious thermal level, the firing resumes with an irregular low frequency, impulse discharge that lasts for many hours [511, 526]. The conjunctival polymodal units have a thermal threshold that is about 2-3^{o}C higher than the cornea and also exhibit the property of sensitization [516].

Response to Chemicals:

Polymodal neurons respond to substances such as acid, hyperosmotic sodium chloride and CO_2 (which is converted to carbonic acid). In the human conjunctiva and cornea the application of CO_2 evokes a sensation of stinging and/or burning pain. The chemical thresholds measured as a response evoked by CO_2 upon the cornea and the conjunctiva has been determined to range from 31% to 55% of CO_2 [263, 515]. The sensation evoked by a chemical stimulus is different from the sensation evoked by a mechanical stimulus indicating a selective response of receptors of the peripheral nerve terminal. Protons stimulate the nociceptive endings and a local decrease in the pH is accompanied by a discharge of nerve impulses whose frequency is proportional to the proton concentration [525]. The site of action of the protons is not established but the chemical stimulation of neurons causes an intracellular Ca+ entry into the nerve terminals resulting in an action potential. This response is believed to be mediated by the non-selective vanilloid receptor channels (VR1) and the acid sensing ion channels. In the rat cornea the capsaicin evoked increase of intracellular Ca2+ in peripheral nerve terminals was completely blocked by the VR1 antagonist capsazepine [484]. Adenosine-5′- triphosphate (ATP) which is normally released from damaged cells or nerve endings when applied to the rabbit corneal epithelium also causes an increase in the intracellular calcium ion concentration. This increase which is especially prominent in the wing cell layers is believed to be via the P2Y receptors and the intercellular gap junctions [527, 528]. Inflammatory mediators released during inflammation or injuries have an excitatory effect on the polymodal neurons indicating that polymodal receptors transmit nociceptive information of inflammatory origin. Bradykinin evokes responses in the polymodal nerves at very low concentrations and is believed to be an "endogenous pain-producing substance" [529]. Prostaglandin E_2 (PGE_2) when applied to the cornea caused a dose dependant response in the Aδ and C polymodal receptors [516]. Substances such as serotonin (5HT) and histamine (HA) do not evoke significant discharges individually although they all cause a long lasting sensitization [469, 513]. The application of a mixture of inflammatory mediators (inflammatory soup) on the cornea evoked a brisk and vigorous electrical response in the nerve [505, 530].

Cold responses:

Polymodal neurons respond only weakly to cold. A low frequency discharge is seen at 29^{o} C and a further decrease to 20^{o} C diminishes the silent background activity of nociceptors [264, 516].

Itch Neurons:

It is now believed that distinct neurons encode the itch sensation both peripherally and centrally [531-533]. Separate itch fibers in the human eye are not yet documented but animal and human studies, ocular symptom surveys and psychophysical studies provide evidence for the existence of nerve fibers that encode the itch sensation. In the cat a separate pathway for the itch sensation has been shown and neurons in the lamina 1 region of the spinothalamic tract which are specifically responsive to histamine have been identified. These second order neurons have thalamic projections (to the lateral thalamus) which are different from those of the nociceptive neurons (which project to the medial thalamus more often) and exhibit distinct conduction velocities [534]. The itch sensation is also distinguished as a distinct sensation in the guinea pig model where the hind-limb scratching response to prostaglandin and histamine induced conjunctival pruritis has been quantified and shown as different from the response to pain and other chemicals [535]. The effects of different chemicals on the subnucleus caudalis region of the trigeminal brainstem were investigated. Single-unit responses of the second order neurons (wide dynamic range (WDR) and nociceptive specific (NS) neurons) were recorded after the application of chemicals to the ipsilateral eye. The dose response relationship revealed that the response to histamine had a rapid onset and a shorter duration compared to capsaicin, acid, and mustard oil [494]. Other studies have shown that the polymodal units which were excited by acetylcholine, PGE1, glutamate and bradykinin did not show a response to histamine [469]. In healthy human subjects the techniques of microneurography (on the cutaneous branch of peroneal nerve) led to the identification of histamine sensitive C-fibers among the slowest conducting mechanically insensitive C-fibers in the lower leg [536]. Symptom surveys involving Dry Eye often note ocular itch as a commonly reported symptom [125, 253, 537]. There is now a mounting body of evidence that a distinct set of fibers encode the itch sensation in the eye.

Second order neurons of the ocular surface:

The stimulation of the Aδ and C fibers activates the second order neurons and different studies have reported the response properties of neurons in the trigeminal brainstem complex. Functionally the second order neurons are classified as low threshold mechanoreceptive, WDR multireceptive responding over a broad range from a lowthreshold mechanoreceptive to noxious stimuli and NS neurons. Electrical responses evoked by the application of chemicals on the cornea in the cat and rat has been measured in the second order neurons in the transition part of the trigeminal subnucleus interpolaris and subnucleus caudalis and in the region between the trigeminal caudalis and the cervical spinal cord (C_1). The units responding exclusively to mechanical units are termed as Class 2 neurons and neurons which respond only to noxious corneal stimuli were termed as Class 3 units. In the interpolaris-caudalis transition, the units from the cornea responded only to a low threshold mechanical stimulus and about 25% of the units were of the WDR type. Units located in the caudalis-C_1 had receptive fields in the cornea and were of the WDR type or NS type [538-540]. These second order neurons also express the protein c-fos after noxious stimulation of the cornea and in the subnucleus caudalis region of the brainstem of the rat these c-fos positive neurons were reduced by the application of Substance P and neurokinin receptor antagonists indicating that Substance P and neurokinin A are involved in transmitting information from the ocular surface to the brainstem [541, 542]. Knowledge about the transmission of information from the second order neurons and the central processing of ocular sensory information is limited. In the cat the brain stem trigeminal ocular neurons project to the contralateral ophthalmic nociceptive specific and the wide dynamic range units located in the nucleus ventralis posteriomedialis of the thalamus. The ophthalmic units in the VPM were defined into three subclasses, nociceptive specific (NS), wide dynamic range (WDR) units and low threshold mechanoreceptive units (LTM) units. These units were located in the dorsolateral margin of the nucleus termed as the shell zone [543]. Thalamic relays project to the primary and the secondary somatosensory region of the orbitofrontal cortex and the anterior cingulate region of the brain. This cortical processing is sustained by reciprocal interactions with the thalamus and the other parts of the brain receiving input from the trigeminal neurons. The electrophysiological responses alone do not explain the

hedonic aspect of discomfort and it is unclear as to how the electrical impulses translate into the various qualities of ocular discomfort. Correlations have been made between the electrical response of ocular units to different stimuli in animal studies and psychophysical responses of human subjects to similar stimuli [264]. As the physiology of the response systems are vastly different, the conclusions drawn from such studies can only be tentative. These electrical and psychophysical responses should be considered in conjunction with the factors affecting the sensitivity of the ocular surface. The sensitivity of the ocular surface is not uniform and demonstrates topographical variations within and between histologically similar and dissimilar tissues. The factors affecting the surface sensitivity are briefly discussed below.

Factors affecting Ocular Surface Sensitivity:

The cornea is one of the most sensitive tissues in the human body. The sensitivity of the apex of the cornea is higher than the peripheral region with regional differences in the peripheral region and the lowest sensitivity being in the superior region [544]. The reason for this increased sensitivity of the apex of the cornea is not clear and is not explained even by the recent findings about the architecture of the nerve terminals [459]. The sensitivity of the ocular surface remains almost the same in persons between the ages of 10 - 50 years. At about 65 years of age the sensitivity falls by half. This decline is attributed to various factors such as increase in the fibrous content of the tissue, lipid infiltration and decreased water content [544, 545]. A significant diurnal variation in the sensitivity of the cornea with low sensitivity in the morning and increased sensitivity in the evening has been reported. The morning fall in sensitivity is attributed to an altered physiology of the closed eye during the sleeping hours [546]. There is no difference in corneal sensitivity between men and women although in women during the period of pre- and during menstrual cycle the sensitivity was noted to be depressed [547]. The sensitivity of the eye was also shown to alter with the color of the eye [548]. Corneal sensitivity is reduced by the use of contact lenses and hypoaesthesia of the cornea is commonly reported after surgical procedures involving the anterior segment. There is often a reduced corneal sensitivity seen in ocular and systemic diseases such as keratoconus, corneal dystrophies and diabetes [549-551]. We do not at present have a satisfactory explanation for the physiological variations in corneal sensitivity

in relation to the neurophysiology of the ocular surface. It is however certain that acute inflammation and an altered physiology of the ocular surface results in markedly increased nerve activity with a spontaneous firing persisting for several hours [516]. The long term ocular surface changes caused by an unstable tear film lead to alterations in the physiology of the integrated ocular surface/lacrimal gland functional unit [552]. These pathological changes of the ocular surface contributed to by an unstable tear film may result in an alteration of the excitability of the multireceptive central neurons. The perception of discomfort caused during a normal tear break up should therefore be measured to improve our understanding of discomfort caused by a drying of the eye. Such a measurement would help provide the baseline data for the immediate and acute discomfort caused by tear drying. As there are no conventional methods for measurement of the intensity and affective dimension of ocular discomfort and ocular pain in real time, a method has been developed to measure the various aspects of discomfort caused by tear drying upon the ocular surface.

EXPERIMENTS:

7. Experiment 1:

The study consisted of two experiments. The first experiment aimed to determine the characteristics of the sensation during transient ocular dryness. This was considered important as the symptoms of ocular discomfort are related to the drying of the eye. The second experiment was developed to overcome the difficulties that were experienced during the first experiment.

Materials and Methods:

Subjects:

9 subjects participated in the study with ages ranging between 25 to 35 years. 3 subjects were females and the rest were males. None of the participants had any regular complaints of ocular irritation. All the subjects completed a baseline ocular examination. None of the participants had any ocular or systemic disorder at the time of participation in the study. One participant had a history of contact lens wear.

Procedure:

A measured quantity of fluorescein dye was introduced onto the surface of the eye. The subjects were instructed to blink and then open the eye and then refrain from blinking for as long as possible. The tear breakup time (TBUT) was determined. Subjects had to indicate the time when discomfort was first perceived. Subjects also rated the quality of discomfort by using sensory or affective verbal descriptors. The site where discomfort was first perceived during the period of eye opening had to be indicated by the forced choice method. For this purpose the ocular surface was divided into the following four quadrants; superior temporal, inferior temporal; superior nasal and inferior nasal. Subjects were also asked specifically about the sensations of dryness and cold on the ocular surface. The subjects were asked to blink if the discomfort was unbearable and the eye could no longer be held open. The blinking resulted in the re-establishment of the corneal tear film. Subjects were again asked to rate the sensation which was experienced soon after blinking. This procedure

was repeated four times in each eye. Measurements for all the subjects were done at about the same time during the afternoon.

Data Analysis:

The data were analyzed by measuring the average time taken for tear breakup and the onset of discomfort. A correlation matrix was generated to note the region of the tear breakup, and the site of discomfort reported.

Results:

The average time for tear breakup from four measurements of each eye, and the corresponding times for the onset of discomfort are reported in Table 7-1.

	RIGHT EYE			**LEFT EYE**		
Subject Number	Average TBUT (secs.)	Onset of Discomfort in seconds	Discomfort time - TBUT time (secs)	Average TBUT (secs)	Onset of Discomfort in seconds	Discomfort time - TBUT time (secs)
Subject: 1	9.00	12.00	3.00	9.00	13.25	4.25
Subject: 2	10.47	12.75	2.28	8.97	10.50	1.53
Subject: 3	11.76	14.65	2.89	11.27	13.00	1.73
Subject: 4	8.25	11.50	3.25	8.83	11.50	2.68
Subject: 5	10.00	12.25	2.25	9.25	13.75	4.50
Subject: 6	10.75	15.25	4.5	10.5	14.75	4.25
Subject: 7	9.25	12.75	3.5	11	13	2
Subject: 8	10.5	11.75	3.25	10	13	3
Subject: 9	8.25	12	3.75	9.25	12.75	3.5

Table 7-1: TBUT and onset of discomfort

A scatterplot relating the onset of Tear breakup time (TBUT) and the time when the discomfort was first reported is shown in Fig 7-1.

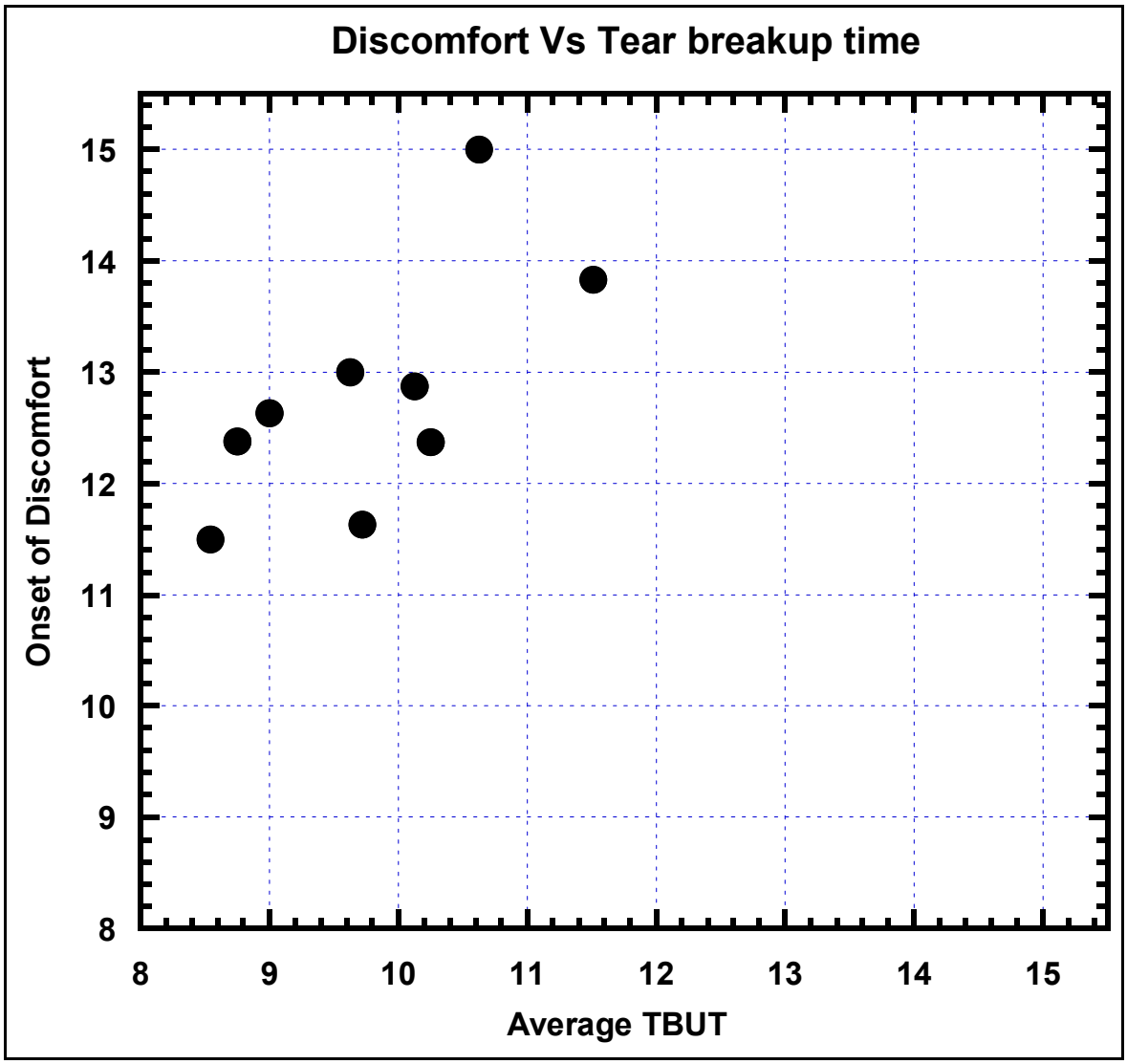


Figure 7-1: Discomfort vs. TBUT

The results of the plot indicate that the tear break up time preceded the onset of discomfort. The subjects also had to specify the region where discomfort was perceived. The responses from the subjects were plotted in a table (Table 7-2 and 7-3). Each correct response marked with the symbol '*', was when the subject reported discomfort in a quadrant where the tear break up was observed. An incorrect response meant that the subjects reporting of the quadrant of discomfort did not correspond to the site of tear breakup observed and this was

marked with the symbol '$_o$' before the response number. Results for each eye are shown separately.

RIGHT EYE										
Accuracy between observed Tear breakup site against Reported Tear breakup site										
		Subject1	Subject2	Subject3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8	Subject 9
Region of Ocular Surface	Sup. Nasal	*3	o1*2o2*3		o4	o1o2*4	o1*3o3			*1o1o4
	Inf. Nasal	*1o1*2o3 *4	*1*4			*3o3o4	*1*2o2*4 o4	*1o1o4	o2	*2*4
	Sup. Temp.		o3	*1*2o2*3 o3*4	*1o1*2o2	*1*2		*2o2*3	*1*2o3*4	*3
	Inf. Temp.	o2o4	o4	o1*4	*3o3*4			o3*4	o1*3o4	o2o3
accuracy %		25%	25%	50%	75%	25%	75%	50%	0%	0%

Table 7-2: RE: Observed tear breakup vs. Reported tear breakup

*** -before the response number indicates the reported site of tear breakup**
o -before the response number indicates the observed site of tear breakup

LEFT EYE										
Accuracy between observed Tear breakup site against Reported Tear breakup site										
		Subject1	Subject2	Subject3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8	Subject 9
Region of Ocular Surface	Sup. Nasal		*2	o3	*2o2	*2o2	*1o1o3	*1o1	*2	*1
	Inf. Nasal	o2	*4	o4*3	*1o1	*4o4	o4	*2o3*4o4	*3o3	o1*3o3*4
	Sup. Temp.	*3o3o4	*1o2o3o4	*1o1*2o2	*3*4	*1	o2*3		*1*4o4	o2
	Inf. Temp.	*1o1*2*4	o1*3	*4	o3o4	o1*3o3	*2*4	o2*3	o1o2	*2o4
accuracy %		25%	25%	50%	75%	25%	75%	50%	0%	0%

Table 7-3: LE- Observed tear breakup vs. Reported tear breakup

*** -before the response number indicates the reported site of tear breakup**
o -before the response number indicates the observed site of tear breakup

The results for the right eye indicate that 22.3% of the subjects' responses were accurate 0% of times. About 33.3% of the responses were accurate only 25% of times. Another 22.2 % of subjects' responses were accurate about 50% of times and only 22.2% of subjects were

accurate in their responses by about 75% of times. The results of the accuracy response for the left eye are shown in table 7-3. The results in the left eye indicate that only 11.0% of the subjects' responses were correct 75% of times. In about 55.5% of responses the subjects identified the site of reported onset of discomfort to region of tear breakup with a 50% accuracy. In about 22% or responses the subjects were accurate only 25% of the times and in 11.1% of responses the subjects were not accurate at all (0%). The results of the quality of the discomfort as rated by the subject are shown below in figure 7-2.

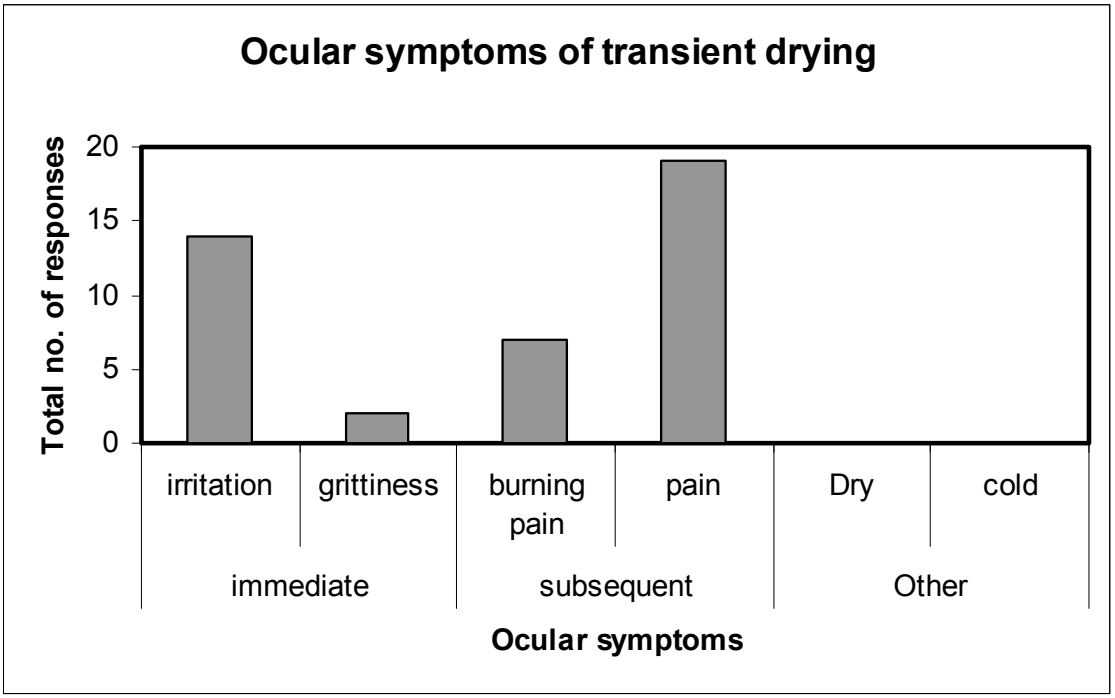


Figure 7-2: Symptoms of ocular drying during forced eye opening

None of the subjects experienced a dry or cold sensation during the time of tear break up. The most frequently reported quality of discomfort caused by the transient drying was irritation. The other symptoms reported were grittiness and burning sensation. All subjects reported that blinking was due to a sensation of pain that was perceived upon forceful opening of the eye. None of the subjects reported a sensation of dry eye or sensation of cold during this period of transient drying of the eye.

Ocular symptoms of transient drying

	Right Eye				Left Eye			
	At onset of discomfort	Sensation causing blink	Dry Eye sensation	Cold	At onset of discomfort	Sensation causing blink	Dry Eye sensation	Cold
Subject 1	irritation	pain	no	no	irritation	pain	no	no
Subject 2	irritation	pain	no	no	irritation	pain	no	no
Subject 3	irritation	pain	no	no	irritation	pain	no	no
Subject 4	irritation > gritiness	pain	no	no	irritation to gritiness	pain	no	no
Subject 5	irritation > burning	pain	no	no	irritation > pain		no	no
Subject 6	irritation > burning	pain	no	no	irritation > burning	pain	no	no
Subject 7	irritation	pain	no	no	irritation	pain	no	no
Subject 8	burning sensation	pain	no	no	burning sensation	pain	no	no
Subject 9	burning sensation	pain	no	no	burning sensation	pain	no	no

Table 7-4: Symptoms of transient drying

Discussion and Conclusion:

The experiment aimed to evaluate the characteristics of the ocular symptoms associated with the disruption of the tear film. The experiment also aimed to understand if subjects could localize the region of onset of discomfort during the tear breakup. Normally the drying of the corneal surface initiates the blink reflex [448]. Dry spots begin to appear on the cornea about 15-30 seconds after a blink and this is believed to be accompanied by a fall in temperature [442, 553]. The tear film break up time due to tear film thinning is modified by enhanced evaporation and humidification [448]. These and other factors influencing the ocular surface activate the corneal and the conjunctival nerve fibers to produce sensations of discomfort, irritation and pain [516]. The quality of the sensations reported by the subjects were mainly irritation, grittiness, burning sensation and pain. Pain was reported as the end event by all the subjects and this is probably because each subject repeated four trails of forced eye opening and the reports of the latter trails may be influenced more by the

chemicals released into the tear film causing an activation of the polymodal and chemosensitive neurons of the ocular surface. These findings are similar to other studies where common symptoms reported after forced eye opening for as long as possible were stinging and burning pain [554]. None of the subjects reported a sensation of cold or dryness. This could be because the thinning of the tear film may not activate significant amounts of cold receptors which are located mainly in the region surrounding the limbus [516]. Alternatively because the subjects were seated in front of a slit lamp the normal drop in the corneal surface temperature that is believed to accompany tear breakup may not have been appreciated in the presence of the slit lamp's illumination with its accompanying heat. Subjects were also not able to localize the region of the tear breakup because a large number of nociceptors are activated by the mechanical and chemical events initiated by forced eye opening causing a suprathreshold sensation event. In the hairy skin the spatial discrimination threshold of painful heat and non-painful touch is 8.6 mm and 9.0 mm [555]. Similar values for the cornea are not presently known and knowledge is limited to the receptive fields of the nerve fibers which have elongated shapes corresponding to the nerve fiber [469]. The poor localization may also be because the receptive fields of the WDR second order neurons receiving input from the trigeminal ganglion have a range of about 1–2 cm^2 and may therefore be incapable of providing stimulus localization [556]. Similar results have been noted in other studies where only about 30-33% of the subjects could identify the localization of the tear breakup [554].

In conclusion this experiment established that ocular discomfort succeeded the tear breakup and the characteristics of the discomfort could be rated. The experiment served to highlight the following difficulties (a) providing responses to multiple types of sensations simultaneously was not an easy task (b) there was often difficulty in monitoring the time event of the subjects' rating of sensations and (c) there was difficulty in noting the exact location of the onset of tear breakup. The second experiment sought to overcome these difficulties.

8. Experiment 2:

The difficulties listed in the first experiment were overcome in the second experiment by using the following method.

Instrumentation:

Description of the "Comfortscope" an instrument for the continuous rating of comfort:

An instrument for measuring the quantitative and qualitative discomfort and pain continuously during tear drying was built at the School of Optometry, University of Waterloo. This instrument which we call "Comfortscope" attempts the bridge the gap in the qualitative and quantitative methods of rating discomfort and pain. The scheme of the instrument is shown in the figure 8-1 below.

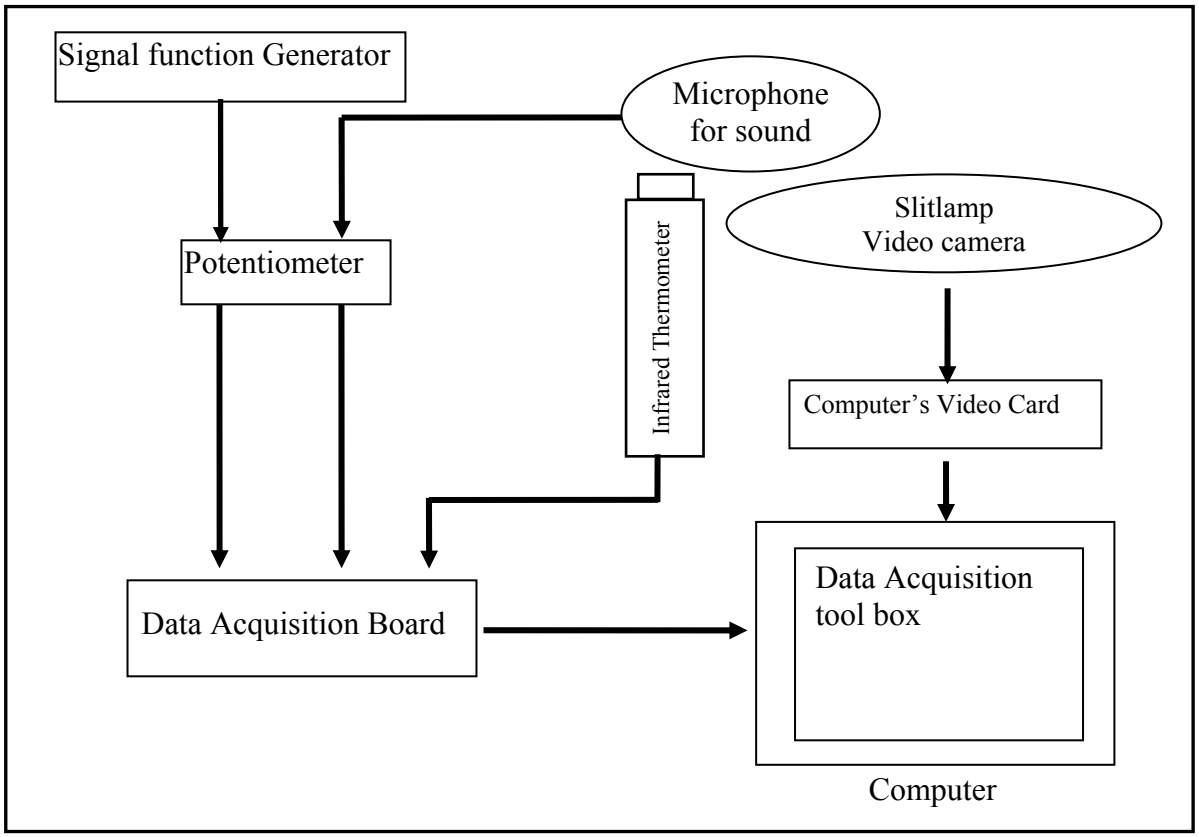


Figure 8-1: Comfortscope Schematic Diagram

The components of the instrument include

1) A signal function generator.
2) A precision single turn rotary dial potentiometer of type A- linear, with carbon composition.
3) A data acquisition card (NI PCI-6035E) from National Instruments.
4) A slit lamp video camera at 5 Hz. to record the ocular changes of the subject.
5) A microphone to record the quality of the discomfort for the affective dimension of discomfort and pain.
6) An infrared non-invasive thermometer (Thi-500 from TASCO) to continuously measure the temperature of the ocular surface.

Software written in MATLAB (Version 5.0) sampled the single turn potentiometer, slit lamp video camera, microphone and an infrared thermometer. The calibration of the instrument was done every time before the recording of data. All the data were collected and plotted on the same time frame at 0.2 second intervals. The scheme for collection of data is shown in figure 8-2. Each trial of rating discomfort due to tearbreakup and drying was recorded into a folder that was automatically created by the "Comfortscope" instrument. A folder at the end of the trial includes:

1) JPEG images of the ocular surface recorded at every 0.2 seconds.
2) A text file named "data.txt" with three columns consisting of
    (i) Time recorded at intervals of 0.2 seconds,
    (ii) Intensity of discomfort corresponding to time and
    (iii) Temperature values obtained from the infra-red thermometer.
3) A file named "data.mat" consisting of sound data.

Subjects:

23 subjects (11 male and 12 female) with ages ranging from 20-30 years, with no symptoms of dry eyes participated in the study. All the participants gave informed consent to a protocol approved by the Office of Research Ethics at the University of Waterloo. None of the

subjects wore contact lenses during the period of the study. To familiarize subjects with the psychophysical rating method all the subjects were trained to use the instrument before data collection.

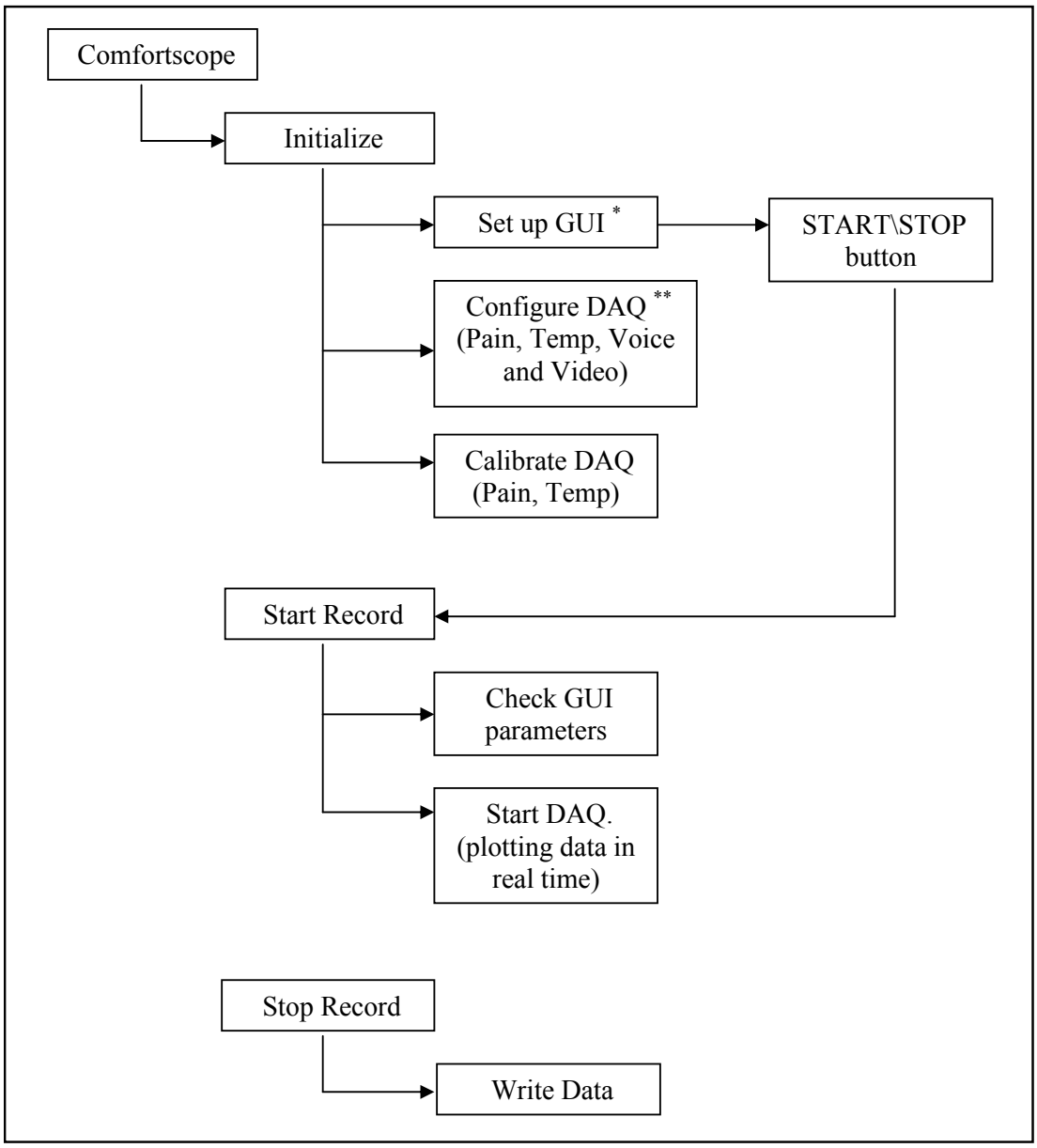


Figure 8-2: Scheme for collection of continuous ratings of discomfort and pain.

* GUI = Graphic user interface

** DAQ = Data acquisition

Psychophysical rating method:

The data from each subject were obtained from three discomfort ratings in one day and this was repeated for three consecutive days. For analysis presented in this thesis, individual data sets that had full scaling, imaging and verbal data were used. The left eye was chosen in all subjects. The right eye was taped during the period of the study. A drop of fluorescein was instilled into the conjunctival sac. Subjects were required to blink once to enable an even spread of the fluorescein and then keep the eye open for as long as possible. The ocular surface appearance and tear film was monitored using a slit lamp. Subjects rated the intensity of the sensation by adjusting the single turn potentiometer to represent the strength of the discomfort. The intensity and characteristics of the discomfort as spoken into the microphone by the subject were recorded. The room temperature and humidity was approximately constant during the period of the study. The calibration of the instrument was undertaken every time before recording data from a subject.

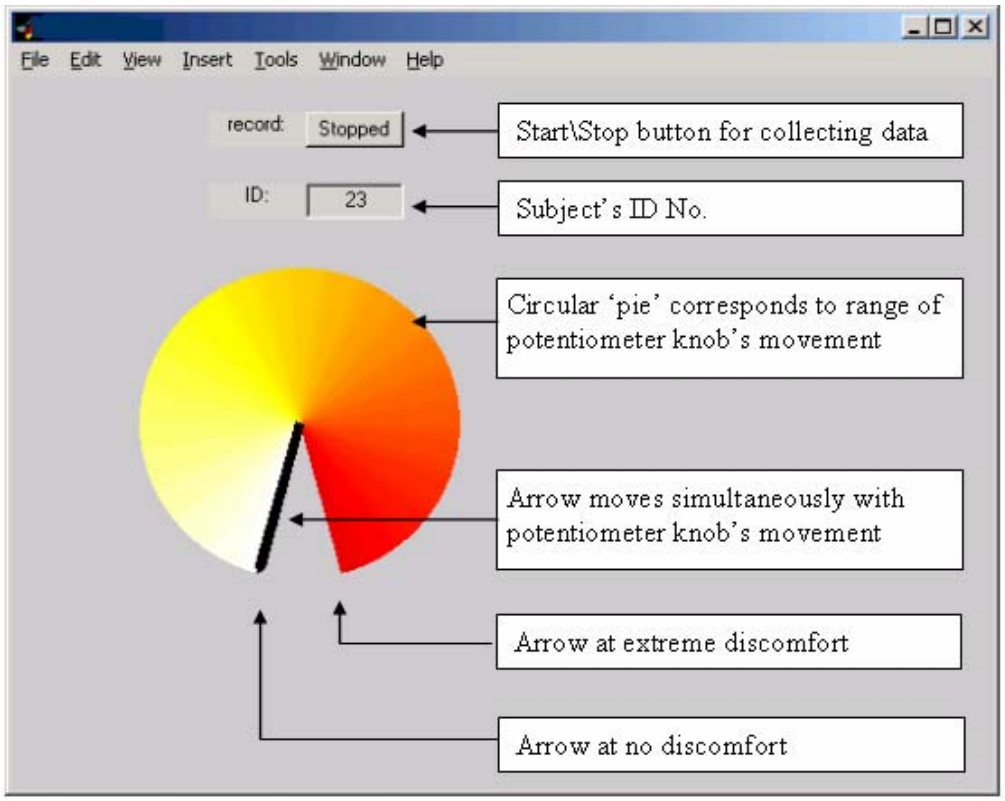


Figure 8-3: Graphic User Interface for data collection created with MATLAB

Two fields are displayed on the GUI. A "record" field consisting of a "Stopped" which changed to a "Started" when clicked upon. An "ID" field to enter the ID number of the subject. The large circular "pie" type of graphic interface corresponds to the range of movement of the potentiometer. The black arrow moves simultaneously and corresponds to the movement of the potentiometer's knob. When the instrument was started for recording the psychophysical rating of quantitative and qualitative data were acquired simultaneously and plotted on the same time frame. All the digital images of the ocular surface obtained from the slit lamp were recorded in JPEG format with the picture quality set at 90 and resolution of 146 x 176. Following data collection a quick preview of the data was displayed as seen in the figure 8-4. This preview was used during training to provide visual feedback to subjects about the potentiometer. During the data collection subjects received no feedback other than kinesthesis related to the potentiometer position.

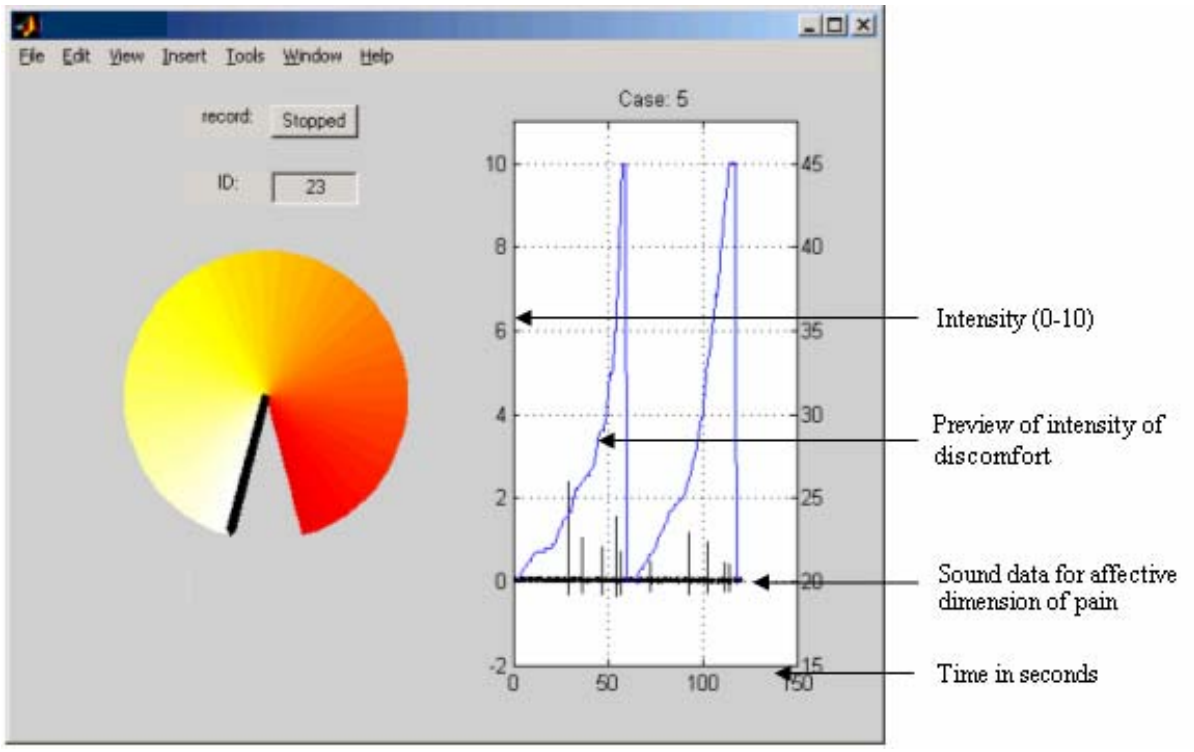


Figure 8-4: Preview displayed when the instrument is stopped.

Analysis:

The analysis of the discomfort data was done in Statistica ’98, with the Non-linear model of curve fitting and by means of a “user defined function”. The video slit lamp images obtained at 0.2 seconds interval were analyzed in ObjectImage 2.08 software. A custom macro was written for the analysis of the images. Irregular illumination and a low quality of the images were important difficulties encountered and overcome during image analysis. The change in the tear film fluorescence indicated by increasing dark areas over the cornea was assumed to represent the drying of the tear film and was measured. All images were converted from JPEG to TIFF format before analysis using “Graphic Converter 4.0”. The custom macro delineated the cornea and calculated this tear drying in the following manner.

1) Each image was opened automatically its title was copied into a variable called "winT" and the green slice of the three stack image (RGB) was duplicated. The duplicated temporary green image was inverted, autothresholded and made binary, which separated the white area of the eye.

2) This binary image of the white area was then subjected to a short series of erosions and dilations which at first removed and then added a single pixel layer and smoothed the image to remove small particles.

3) Then the image was automatically outlined (i.e., outlined with a "marching ants" selection) and the perimeter was calculated, giving every point along the perimeter in x-y coordinates. The top-most left-hand and top-most right-hand points were determined and a curve was created to mimic the curve of the upper eyelid. This step created a close approximation of the corneal area in each image (figure 8-5).

4) Next this corneal region of interest (ROI) is created in the undisturbed image using the "restoreROI" feature and the average mean of this area is measured and stored in a user-defined array. Also stored at this stage were the coordinates of the bounding rectangle of this cornea estimation.

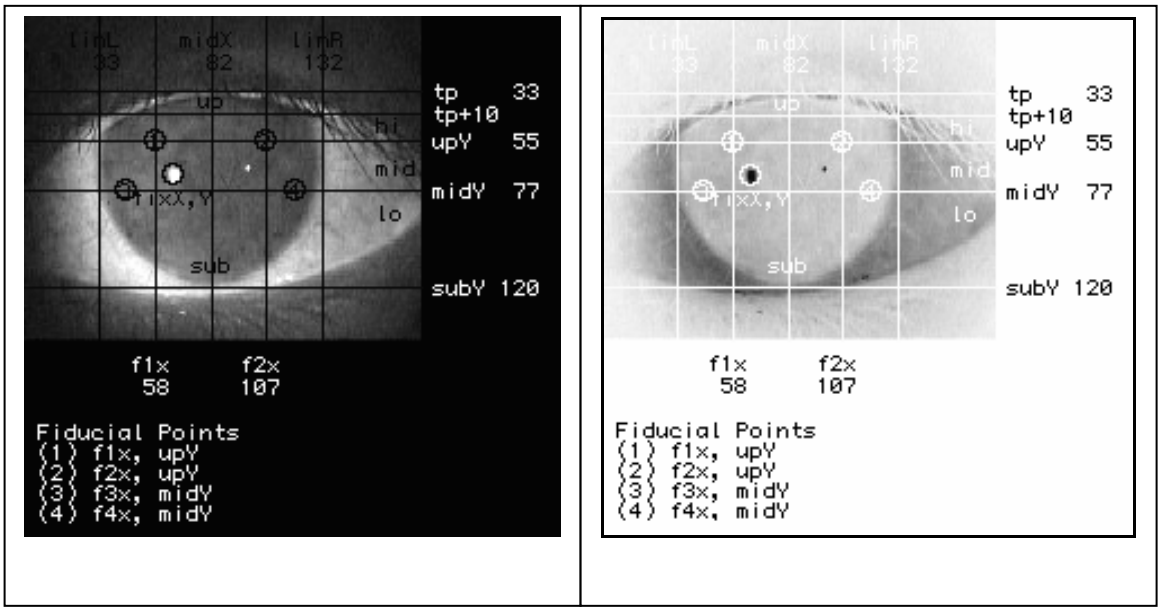


Figure 8-5: Automated image processing to determine the extent of tear drying.

Green slice of the RGB image is duplicated, then inverted and autothresholded (lighter image on the right side) and made binary to be smoothed. After a series of erosions and dilations the binary image is automatically outlined to create an approximation of the corneal image. After further processing the perimeter is calculated and this overlay is used in the undisturbed cornea to delineate the region of interest.

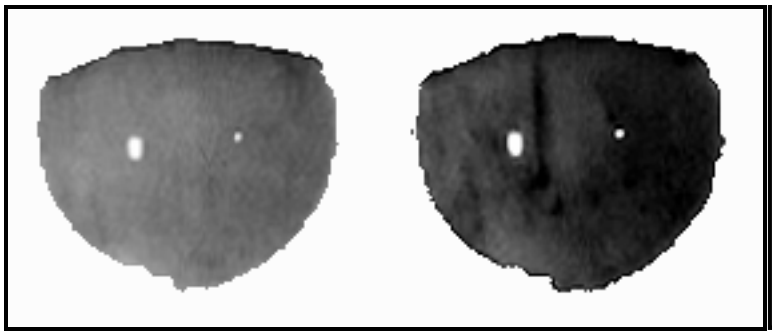
5) After all the images were processed in this fashion, the mean area of all the images was called from the statistical workup of the mean data and the largest bounding rectangle was determined.

6) The oval ROI used to measure each image in a series was taken from the maximum coordinates of the bounding rectangles in each image of the first series, i.e., the left most, top most, right most, and bottom most coordinates of all the images determined the left, top, width, and height of the ROI of the cornea.

7) The area of this ROI was the total area (which is almost constant for a given series of images) and the total and mean grayscale level of each corneal ROI was determined (figure 8-6).

Data of the symptom characteristics were analyzed in a program termed “Painview” written in MATLAB. Linear markers were placed at the beginning and end of the sound wave

displayed by “Painview”. The spoken characteristics recorded were replayed by clicking on the play button (figure 8-7) and the exact time of the spoken event was noted.



The two images represent appearance upon eye opening and just before blink.

Figure 8-6: Example of automatic outlining of the cornea

Results:

A sample of multidimensional data obtained during a measurement is shown in figure 8-7. The intensity of the ocular discomfort which results from varying lengths of dryness of the eye can be recorded immediately. The affective unpleasantness and the characteristics of the discomfort are recorded on the same time scale.

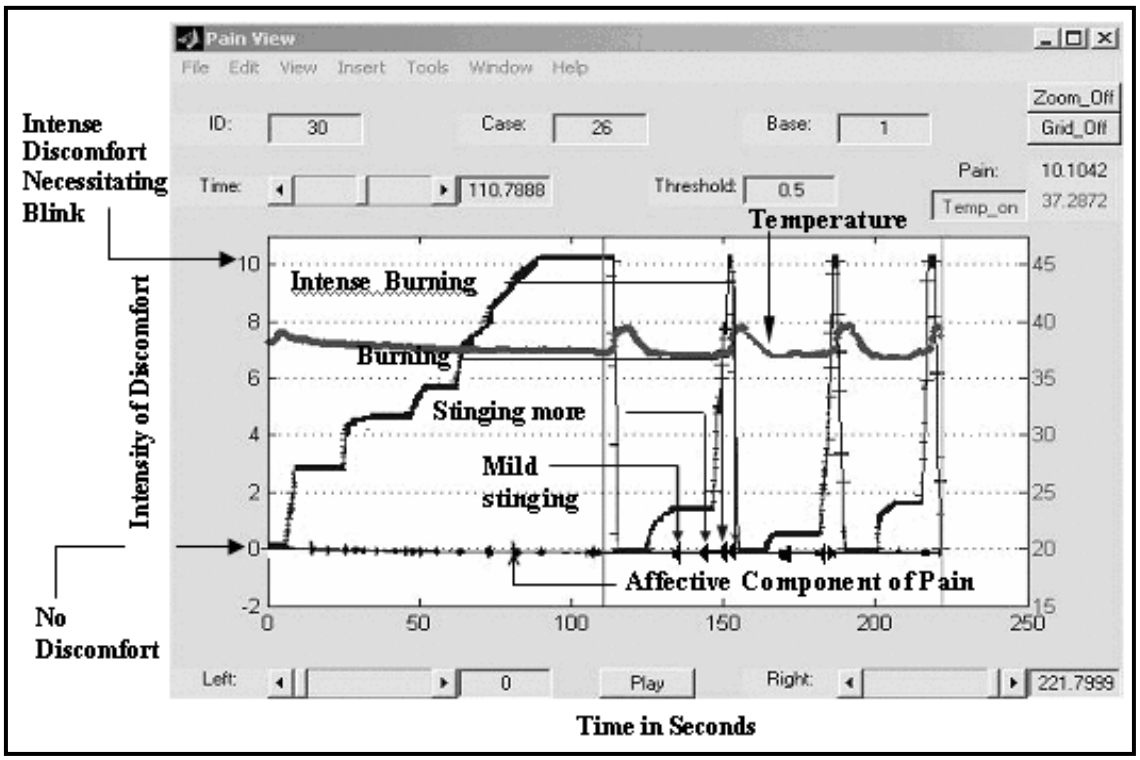


Figure 8-7: Sample of Multidimensional data obtained simultaneously

Results -Ratings of Discomfort:

An analysis of the results showed that the intensity of the discomfort preceding blink exhibited at least three different phases. There were also distinct patterns in the discomfort ratings. In 67.65% of the subjects the intensity of the sensation which preceded blink was triphasic in nature. Upon opening the eye there was a brief period with no alteration in the nature of the sensation termed as the "no change" phase. This was reflected on the scale as the period of no change in discomfort intensity during the initial few seconds. The second phase was due to the "slowly rising phase" of discomfort and the slope of this phase was less steep. The third phase was due to a "rapidly rising phase" of discomfort and was characterized by a more steep slope (figure 8-8 and 8-9).

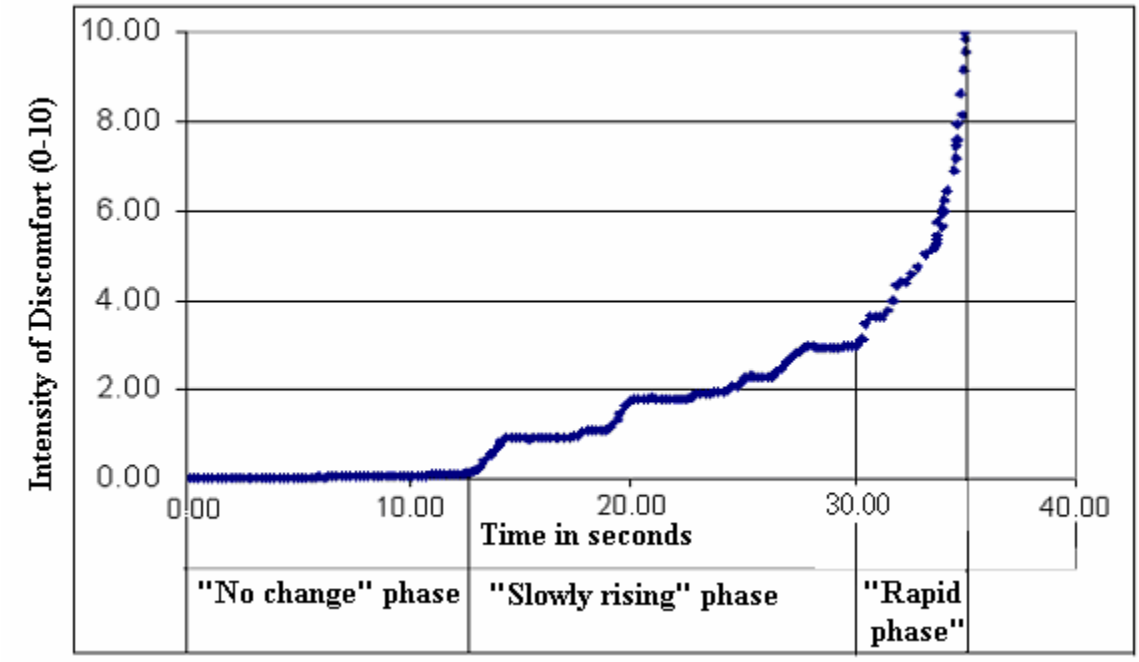


Figure 8-8: The typical triphasic pattern of discomfort

The three phases are an initial "no change" phase of discomfort followed by a second "slowly rising phase" and the third "rapidly rising phase" of discomfort.

Two linear functions with a variable "elbow" position described the data well with correlation coefficients typically of at least 0.95. An example of the linear functions describing the data is shown in figure 8-10.

A correlation analysis between the clinical tear breakup time (TBUT) and ocular discomfort was done and the results are shown in the Table 8-1.

The results in the table show that:

1) The clinical TBUT is inversely correlated with the slope
2) The shorter the TBUT the steeper is the slope
3) The steeper the slope the more rapid is the increase in discomfort.

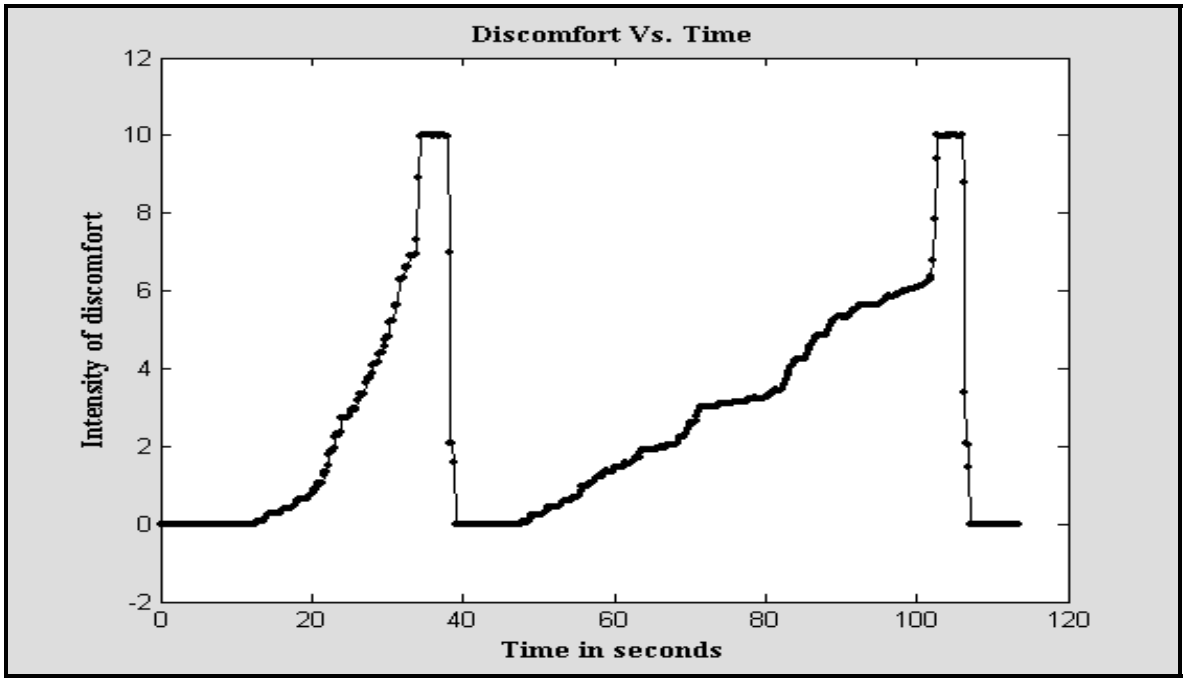


Figure 8-9: Typical discomfort pattern showing the three phases

The data above represents two consequent interblink intervals.

The correlation between the TBUT and the elbow determined by the function indicated that the shorter the TBUT the quicker the onset of discomfort and this is shown in figure 8-11. The correlation between the two slopes is that the steeper the slope of the initial phase the steeper the slope of the second phase and the longer and flatter the slope of the initial phase the longer and flatter the slope of the second phase. This is shown in the steep functions in figure 8-11 and 8-12 and the longer and less steep slopes in figure 8-13. In 17.28 % of the

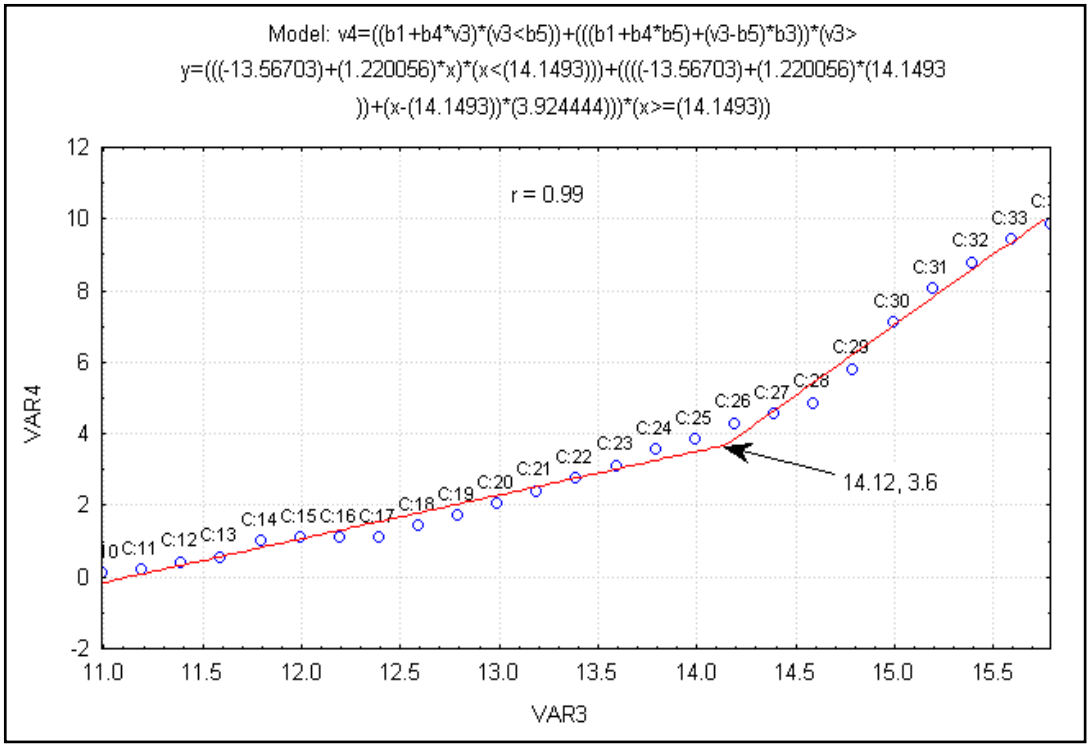


Figure 8-10: Two linear functions describe the data.

The functions described the data with correlations typically at least 0.95

	Correlations: N = 23, Marked correlations are significant at p<0.05					
Variable	**TBUT**	**START**	**BREAK**	**ELBOW**	**SLOPE 1**	**SLOPE 2**
TBUT	1.0					
START	0.41	1.00				
BREAK	**0.74**	**-0.70**	1.00			
ELBOW	**0.72**	-0.25	**0.87**	1.00		
SLOPE 1	**-0.67**	-0.40	**-0.69**	**-0.65**	1.00	
SLOPE 2	**-0.47**	0.01	-0.37	**-0.57**	**0.45**	1.00

p < 0.05

Table 8-1: Associations between TBUT and Ocular Discomfort

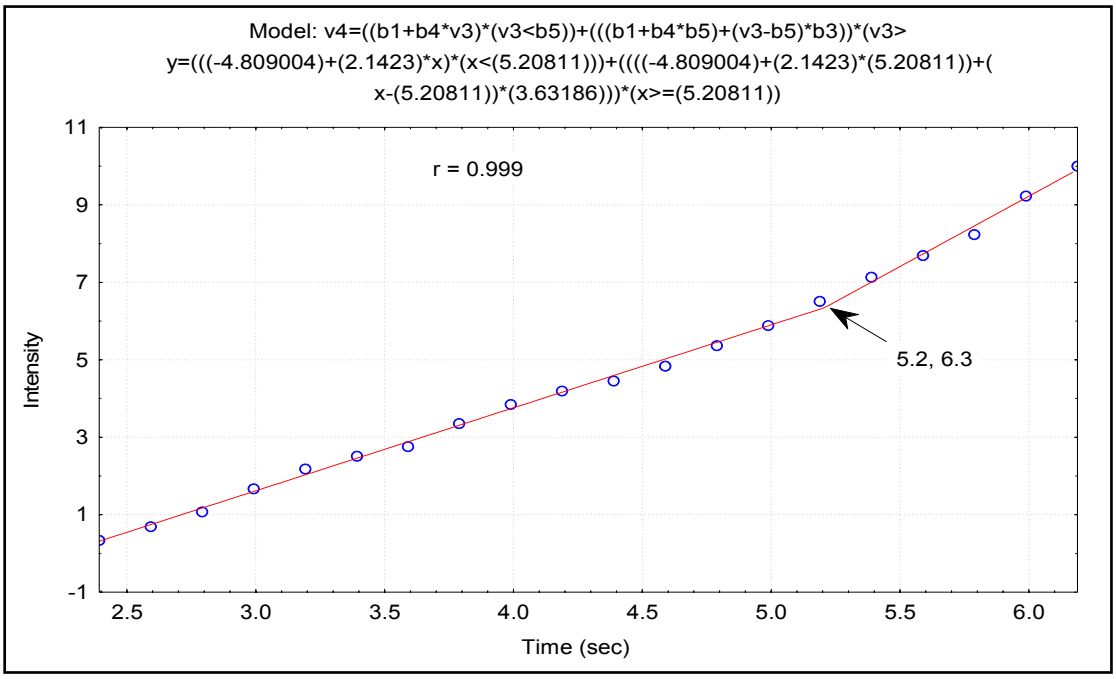


Figure 8-11: Graph indicating that a short TBUT is associated with a steep slope and a rapid increase in discomfort.

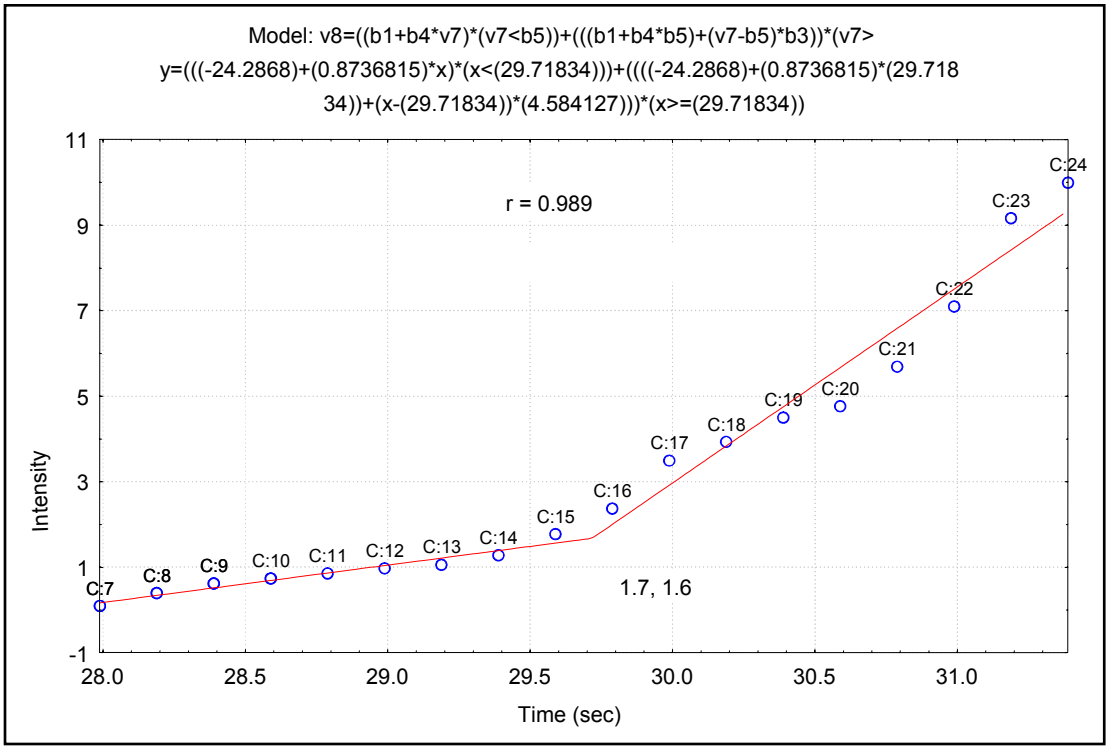


Figure 8-12: Correlation between the TBUT and the elbow is indicative that a short TBUT is associated with a rapid onset of discomfort.

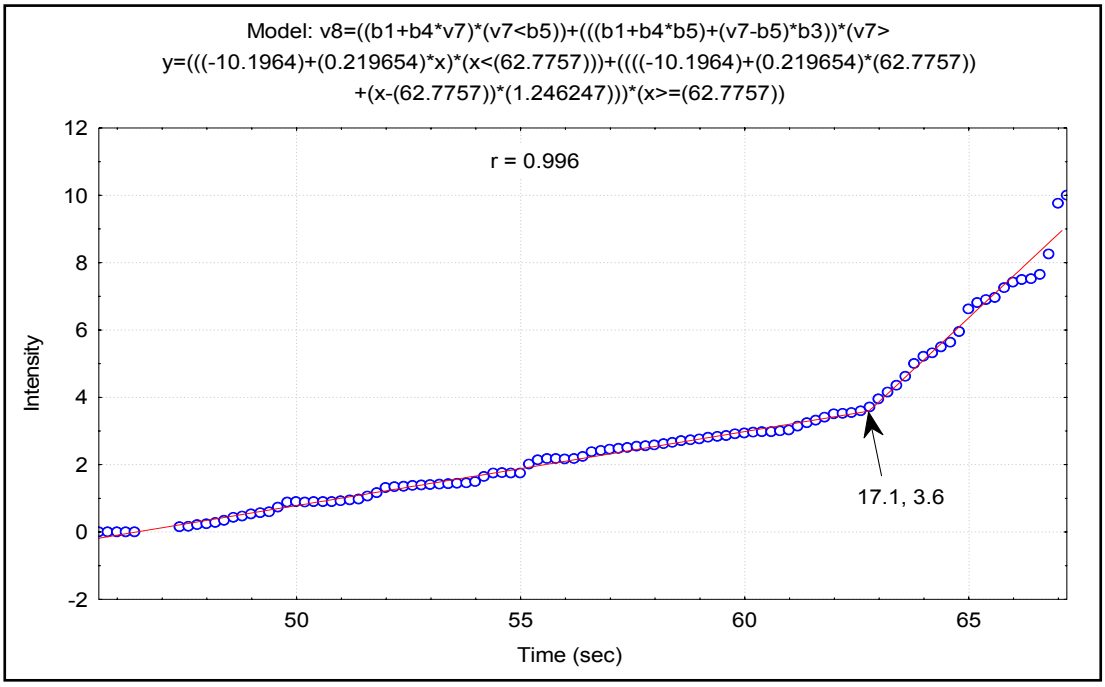


Figure 8-13: The less steep the slope of the first phase the less steep the slope of the second phase.

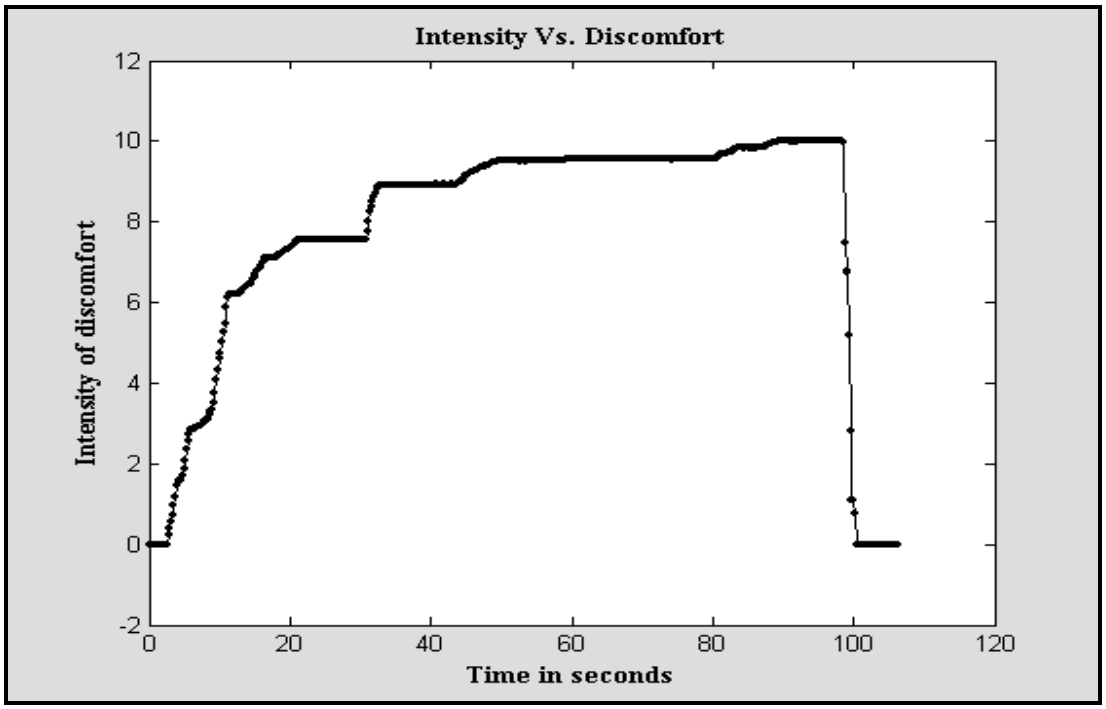


Figure 8-14: An example of atypical triphasic pattern of discomfort with initial "no change" followed by a rapid phase and a subsequent less rapid phase of discomfort.

subjects there was an initial phase where the discomfort increased rapidly and this was followed by a phase of slowly increasing discomfort. This reverse type of atypical pattern is shown in figure 8-14. A mixed response consisting of a typical pattern in the first psychophysical rating followed by an atypical pattern in the second rating or an atypical pattern in the first psychophysical rating followed by a typical pattern in the second trail was seen in about 7.65% of the ratings. An incomplete response where the subject was not able to rate the discomfort due to an inadvertent sudden closure of the eye was seen in about 7.41% of the ratings.

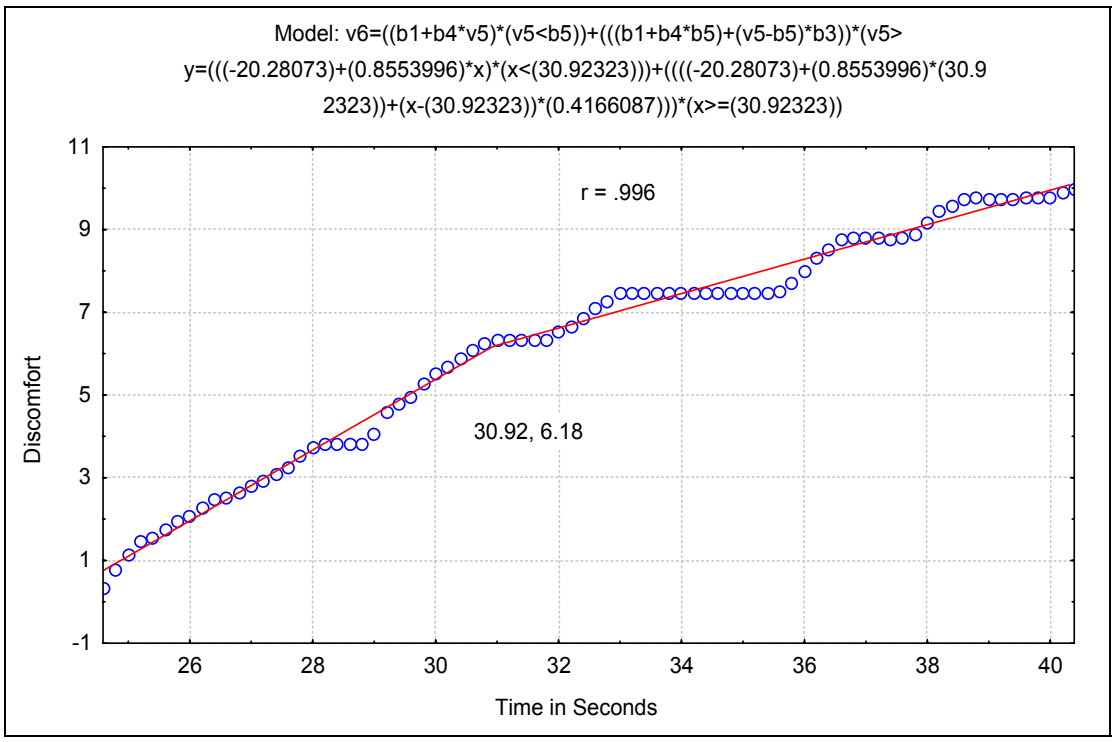


Figure 8-15: Two linear functions describe the data with atypical pattern of discomfort

Results –association of tear breakup with discomfort:

Analysis of the relationships between the ratings of ocular discomfort and extent of dry areas as seen by the fluorescein patterns showed a monotonic association with linear r^2 of at least 0.80 (all $p < 0.05$). The associations with the typical and atypical pattern of discomfort are shown in figures 8-16 and 8-17. In other instances the tear drying consisted of the atypical

pattern with an initial rapid phase of drying followed by a more gradual phase of drying and the corresponding psychophysical rating of discomfort was typical in pattern (figure 8-18).

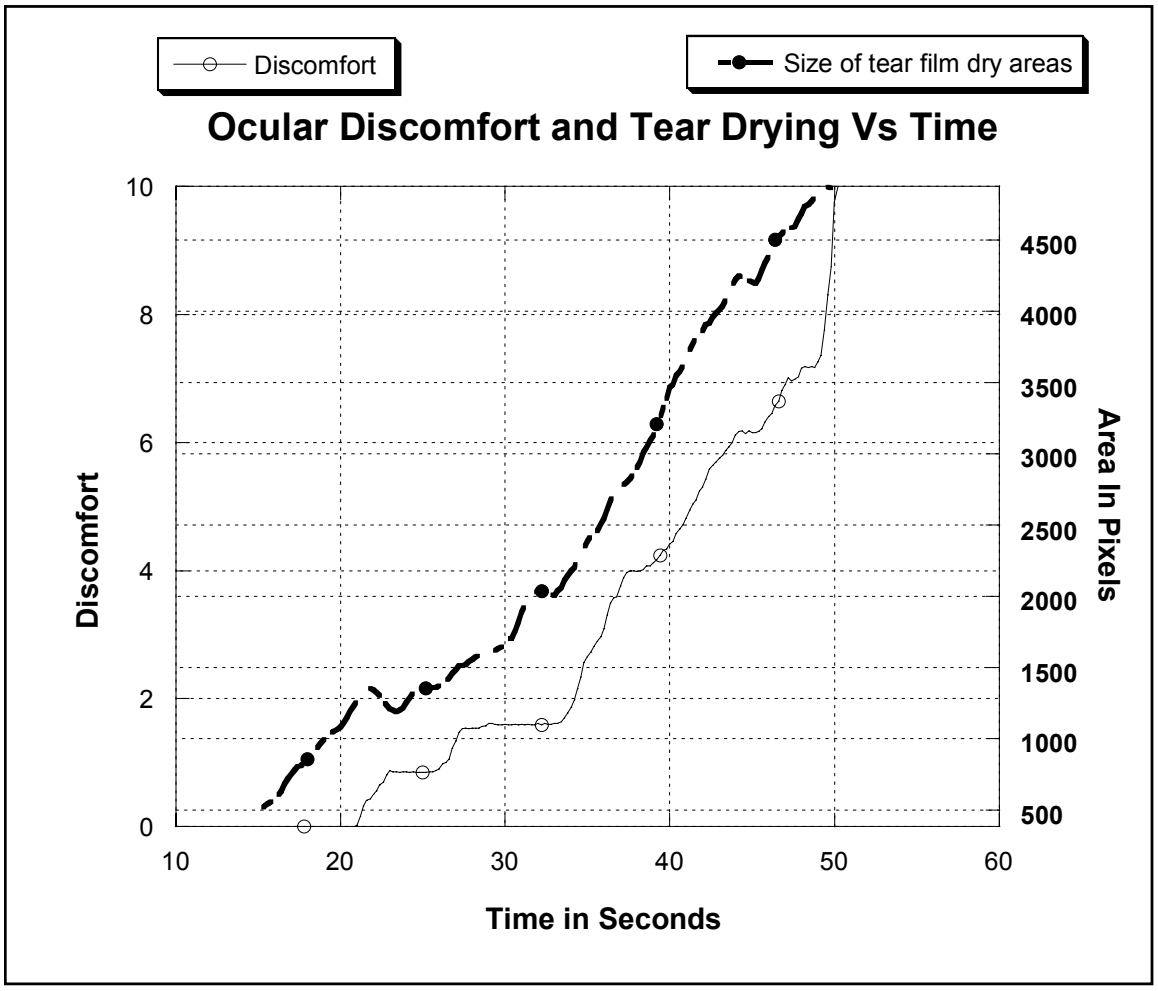


Figure 8-16: A monotonic association can be noted between tear drying and ocular discomfort with initial slow phase of discomfort followed by a rapid increase in discomfort. This is the typical pattern of discomfort.

The two linear functions with a variable "elbow" position which were used to describe the intensity of discomfort were also used to describe the tear drying on the ocular surface. When the "discomfort elbow" was plotted against the "image elbow" with single data pair for each subject the correlation between the discomfort elbow and image elbow was 0.93 and the changes in dry areas often preceded the ratings of discomfort (figure 8-19).

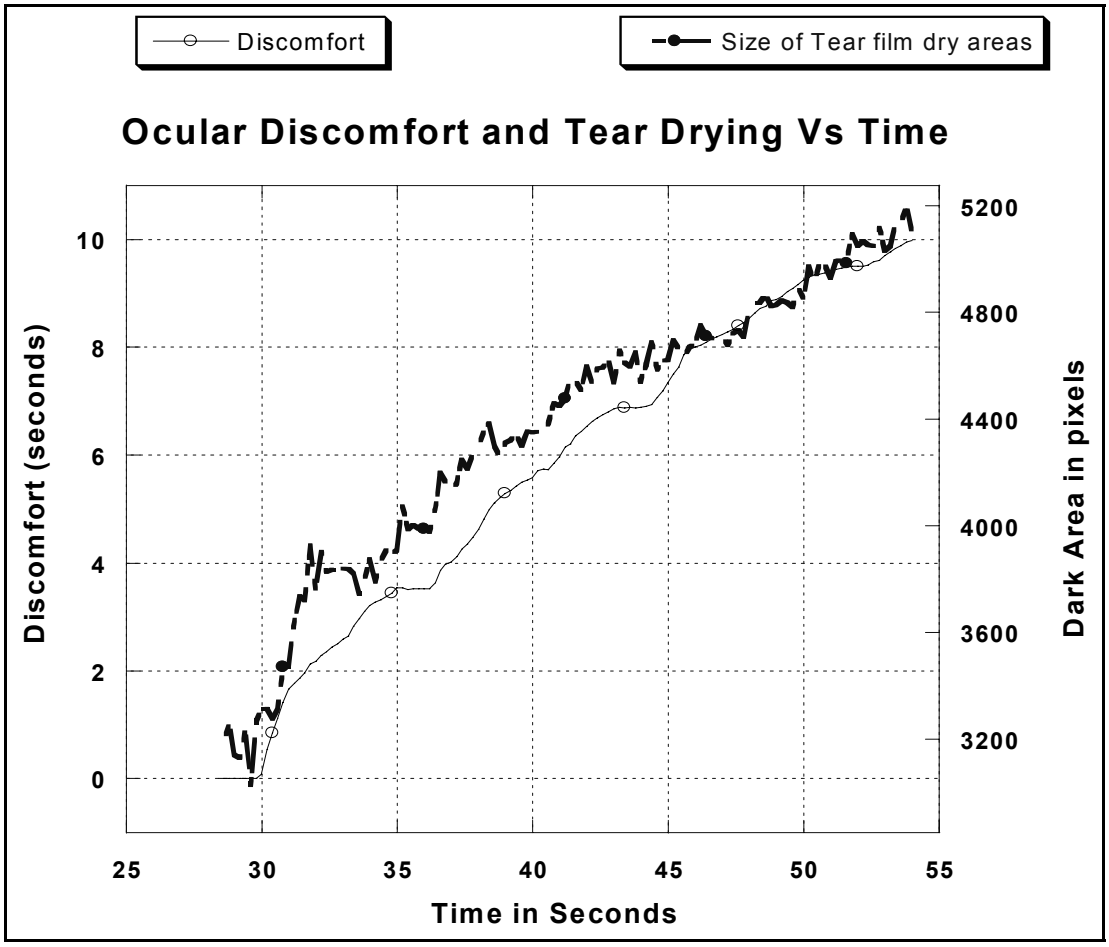


Figure 8-17: An example of association between the tear drying and ocular discomfort with initial rapid phase followed by a subsequent slow phase. The tear drying and intensity rating are both atypical

Results: Symptom Characteristics:

The characteristics of the discomfort recorded into the microphone in "Comfortscope" was replayed in the "Painview" program and the time of the event was noted. Another "multidimensional data preview" function termed "Breaks", written in MATLAB enabled the intensity of discomfort, the ocular surface appearance and the characteristics of the discomfort to be plotted simultaneously. Fiducial markers were displayed in this preview where the regions of changing intensity of discomfort in time could be noted. Clicking upon the fiducial markers displayed the image of the ocular surface. The changing characteristics of discomfort were also plotted in time (figure 8-20).

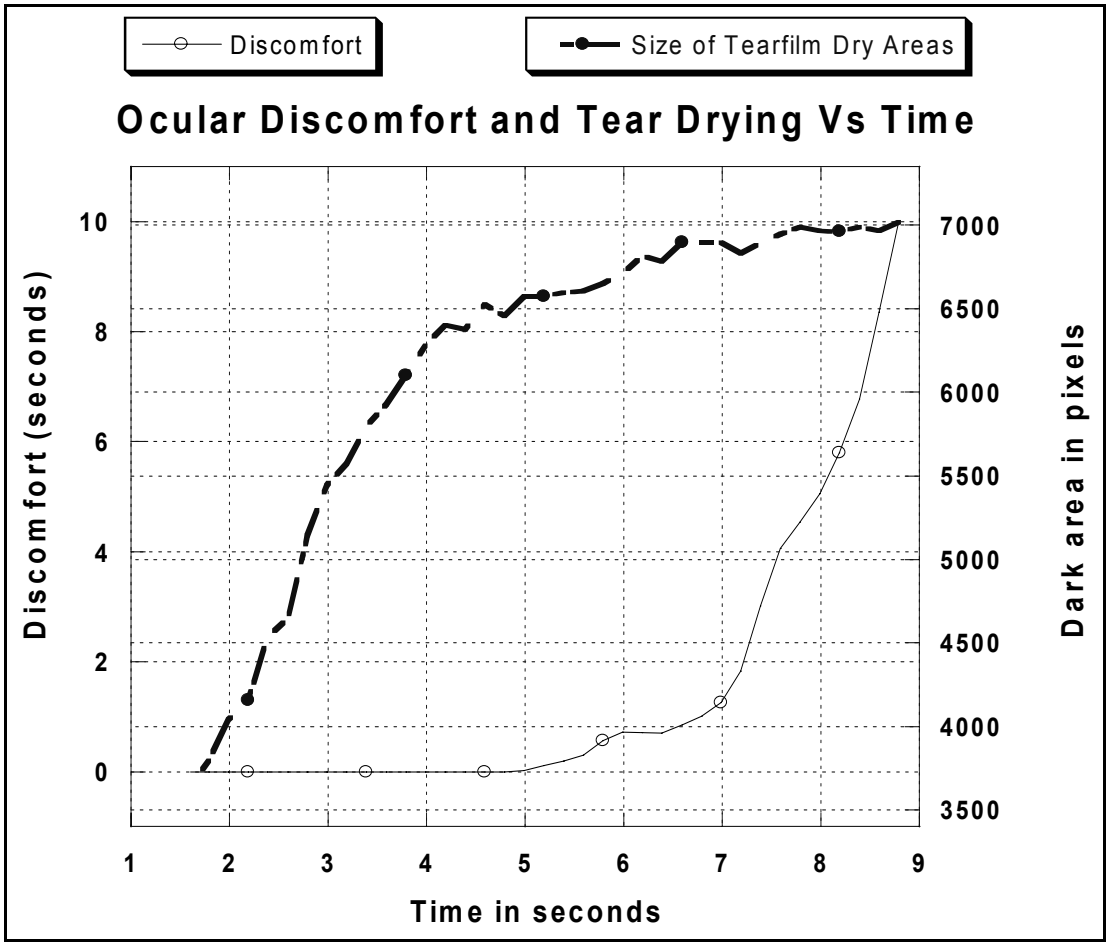


Figure 8-18: Association of atypical pattern of drying and typical pattern of rating of intensity of discomfort

The characteristics of discomfort and pain recorded in the subjects responses were classified into three broad categories

1) mechanical symptoms such as scratchy and dry

2) chemical symptoms that included stinging and burning and

3) itch symptom

The symptom of itch occurred in 29% of the reports during the initial period of the interblink interval. Discomfort associated with the mechanical symptoms occurred in 39% of the reports at the beginning and mid-period of the interblink interval and in only 9% of the reports mechanical symptoms were reported at the end of the interblink interval. In 91% of

the reports chemical symptoms were reported at the time immediately prior to the blink (figures 8-22 and 8-23).

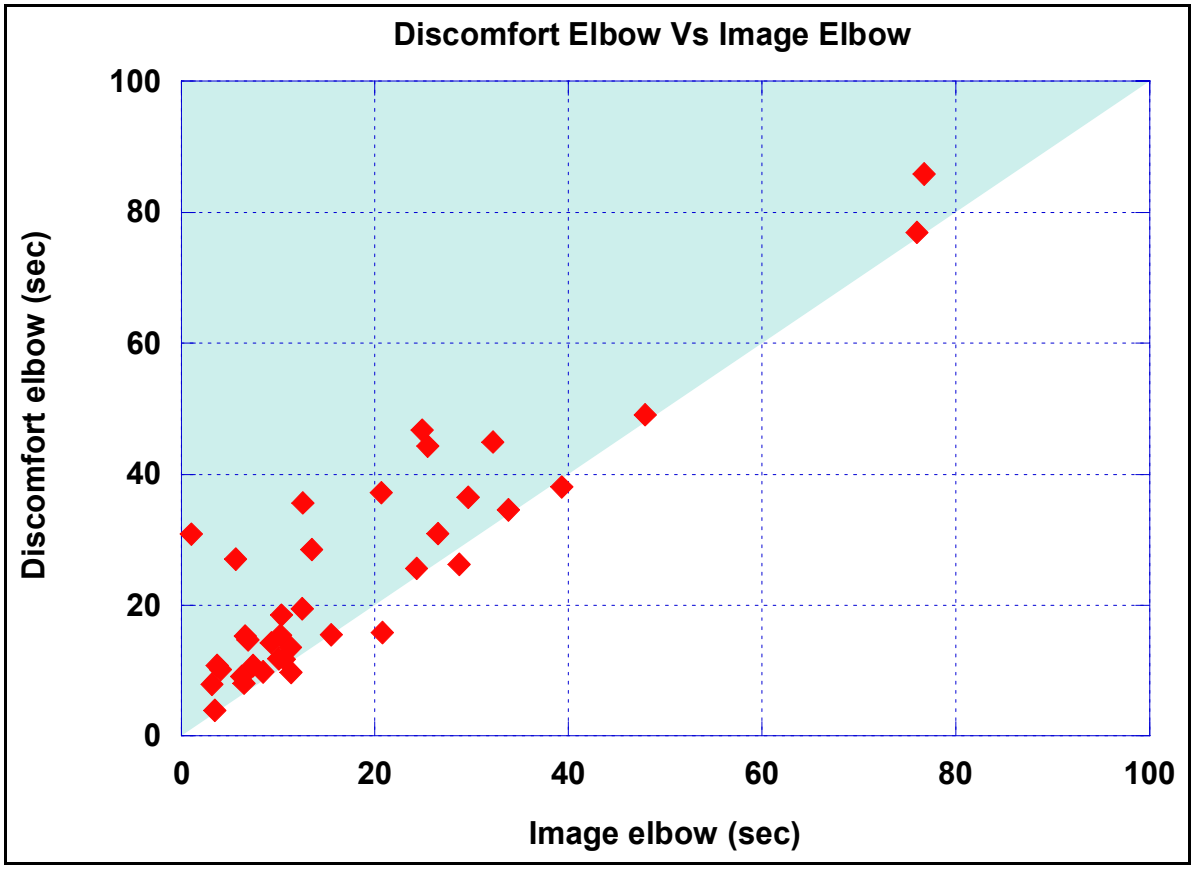


Figure 8-19: The "discomfort elbow" was plotted against the "image elbow" and this revealed that the changes in the dry areas often preceded the ratings of discomfort.

The changing characteristics of the discomfort can be noted in the plot shown in figure 8-21. The symptoms of discomfort were plotted against the intensity of discomfort as shown in figure 8-24. Symptoms of irritation, dryness and itching were noted at the lower range of the intensity of discomfort while symptoms such as stinging, burning and sharp pain occurred at the upper range of the intensity ratings.

"**Breaks**" determined the more local changes in the intensity of discomfort. Local alterations mainly consisted of small fluctuations of rising uncomfortable phases that were followed by less uncomfortable moments. The function determined these changing moments and enabled viewing of the corresponding image and the symptom characteristic if spoken by the subject.

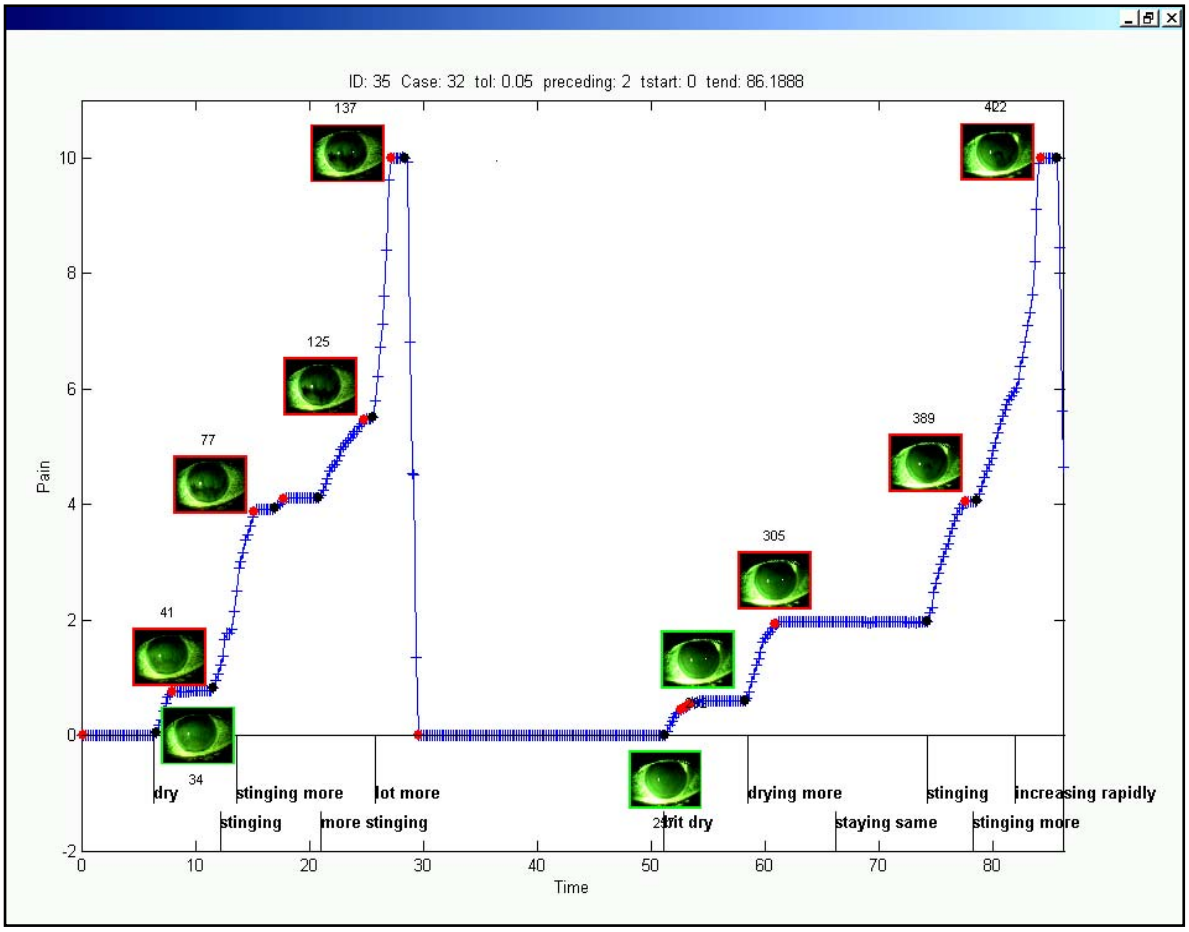


Figure 8-20: The multidimensional data preview function "Breaks"

This function enabled visualization of discomfort, ocular surface appearance and characteristics of the discomfort at the same time. Fiducial markers were displayed by the function at locations of change in the state of discomfort intensity.

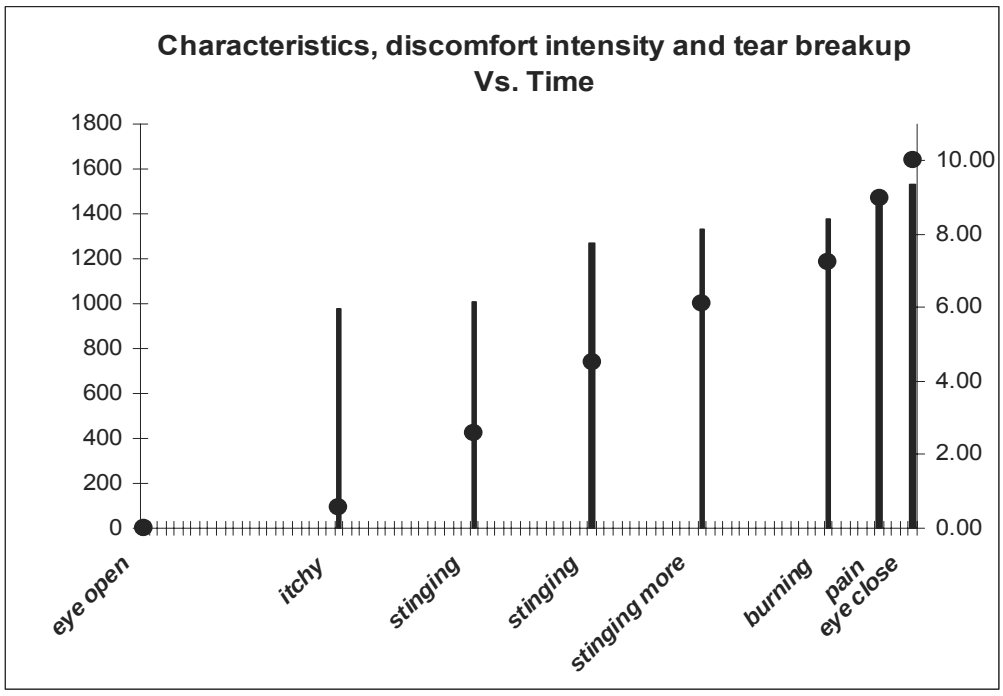


Figure 8-21: Example of data in an individual illustrating the changing characteristics of the discomfort as well as tear film and intensity variables.

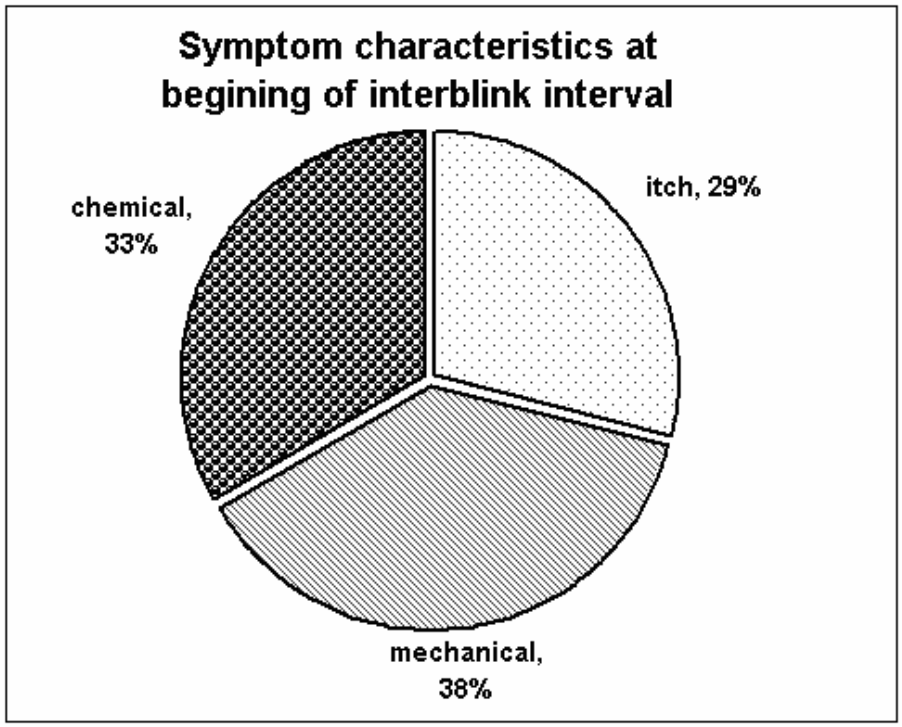


Figure 8-22: Symptoms characteristics at the beginning of the interblink interval.

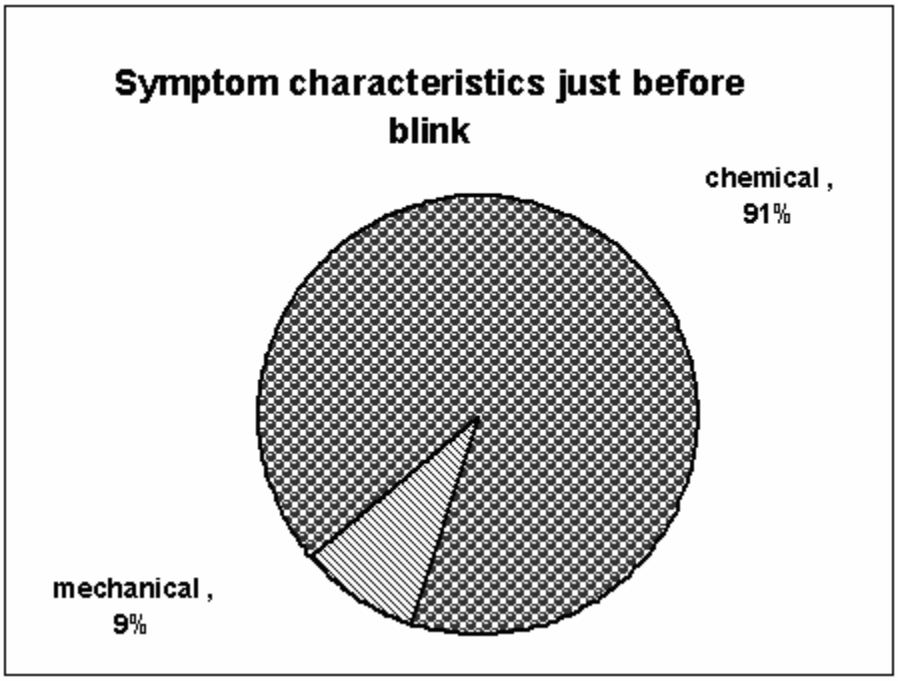


Figure 8-23: Symptom characteristics of discomfort just before blink.

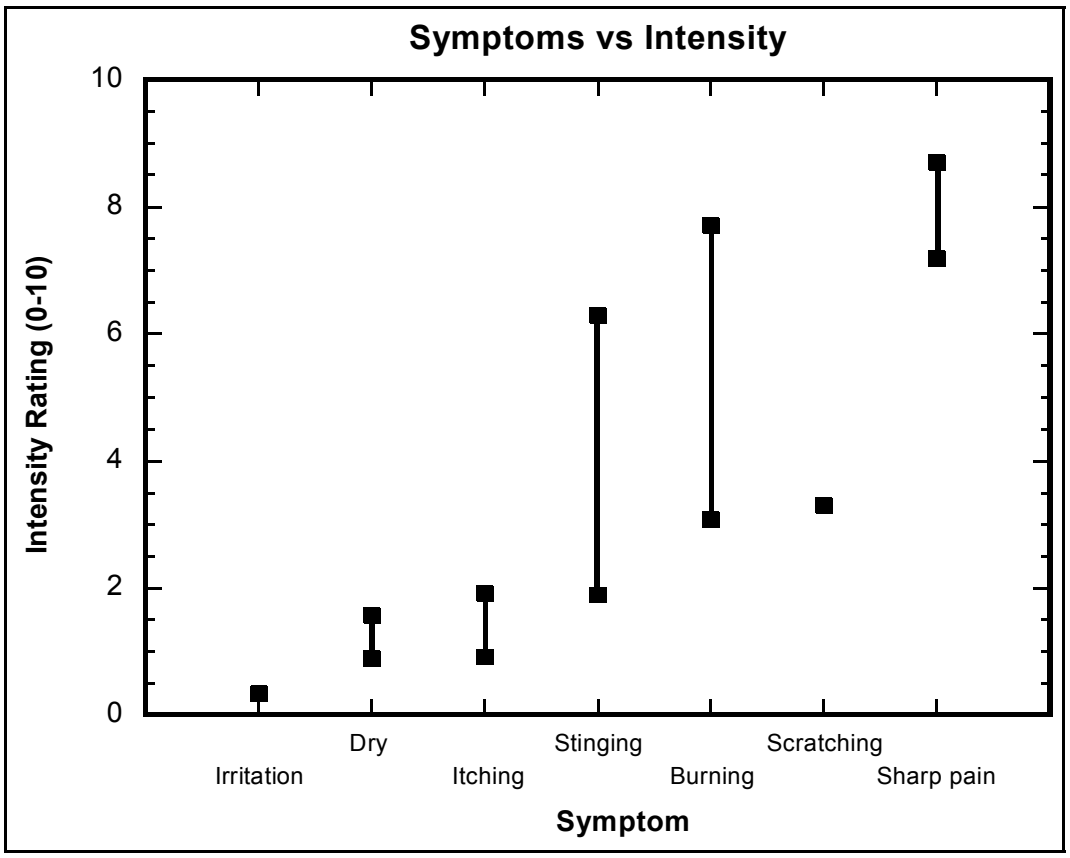


Figure 8-24: Graph shows the relationship between characteristics and intensity ratings

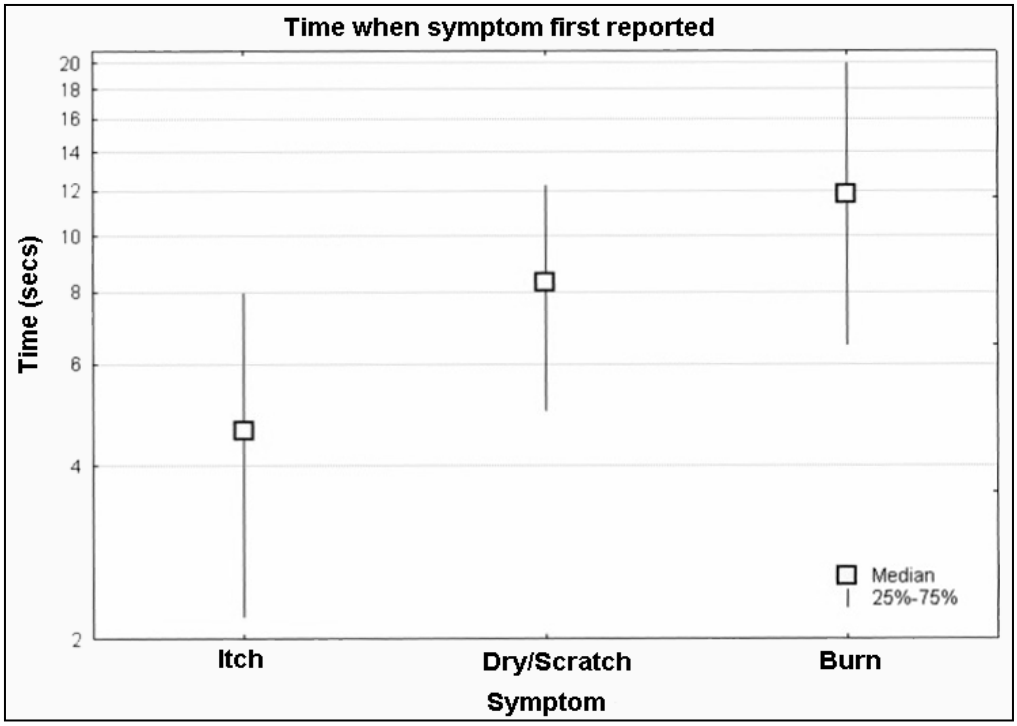


Figure 8-25: Graph showing relationship between symptom characteristics and time

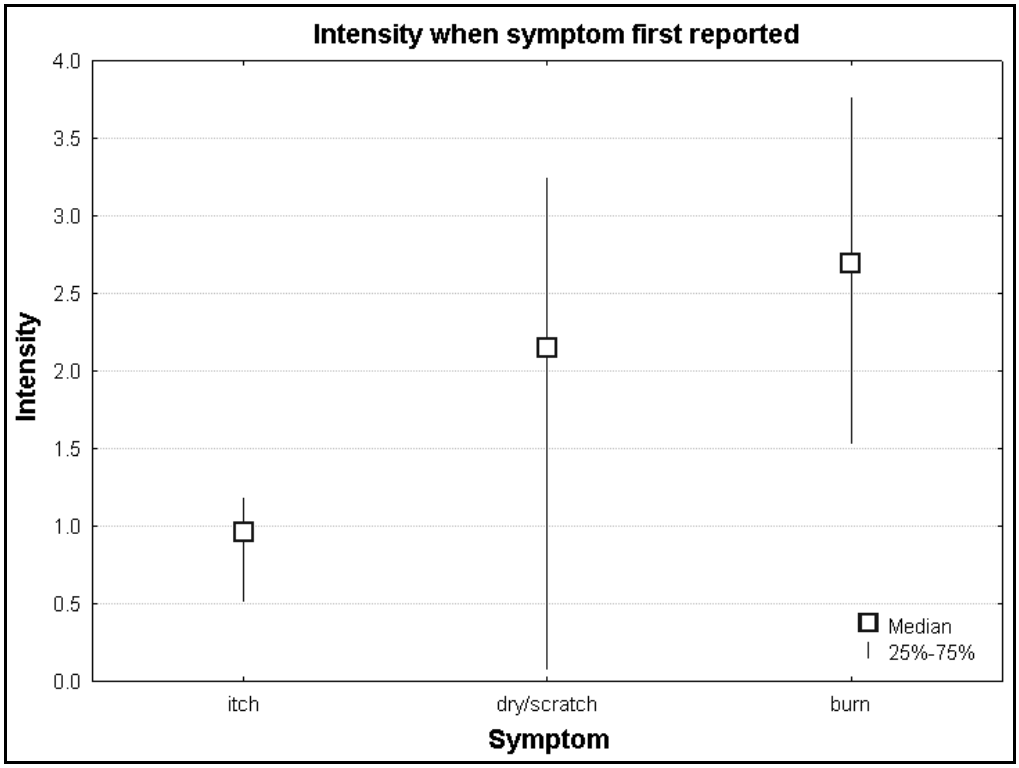


Figure 8-26: Graph shows the relationship between symptom characteristics and Intensity

Figures 8-25 and 8-26 show the intensity and time of first report of the three groups of symptoms illustrating the difference between the three. Kruskal-Wallis analysis showed statistically significant differences between itching and burning for both intensity and time ($p = 0.03$ and $p = 0.02$ respectively).

DISCUSSION AND CONCLUSION:

9. Discussion:

Ocular discomfort associated with dry eye is one of the most commonly reported symptoms in clinical practice [1]. Relief from discomfort has remained an important strategy in the management of dry eye. Clinicians and researchers have always viewed that an essential element of treatment is to reduce the severity of symptoms. It appears that the measurement of this change should be addressed by research methods. The measurement of change has at least three different objectives.

1) To measure differences in the amount of change both within and between individuals and to understand if the change is “little” or a “lot”.
2) To identify the factors that are correlated with this “little” or “lot” of change.
3) To infer the effects of any intervention.

The traditional approaches to the measurement of discomfort and pain include verbal and numeric rating scales, behavioral observation scales and physiological responses. Because discomfort and pain are subjective a patient’s report is considered as the most valid measure of these experiences [557]. Some of the frequently used self rating instruments are the Verbal category scales, Visual analog scales (VASs) and the MPQ. These methods were developed for the discrete rating of discomfort or pain and are employed by practitioners in tear film and dry eye management to assess the characteristics, prevalence, frequency and severity of ocular surface symptoms. These scaling methods are inadequate to measure the changing state of an acute continuous discomfort. The Verbal category and VASs are unidimensional rating scales especially useful to measure one dimension of discomfort or pain. Recent knowledge that pain is no longer a linear system stresses the need for multidimensional scaling methods. The MPQ has been used to assess the multidimensional nature of ocular discomfort caused by tear drying [554]. The disadvantage in employing these methods is that very little information about the physiological or pathological correlates of change is obtained while rating acute either acute pain in general or ocular discomfort in specific. Various studies have used different methods to capture information about the events that cause a change. Some of these methods attempted to eliminate recall

bias and initially paper diaries and subsequently electronic diaries were introduced to instantly record information about brief intervals of discomfort and pain. Either non-compliance or a low compliance with electronic and paper diaries remained a major problem for recording acute events [558, 559]. Recently computer-based Visual analog scales (VAS) and affective scales were used for the psychophysical ratings of discomfort and pain. The two rating scales (VAS and the affective scale) were displayed sequentially and subjects used a computer mouse to rate discomfort [279]. Others studies have combined brain imaging with psychophysical methods to understand the sensory and affective dimensions of pain [560, 561]. In the study of ocular discomfort the main methods employed are symptom surveys, clinical tests for determining the tear secretion rate and staining techniques to estimate the epithelial damage. Instability of the tear film is implicated in causing symptoms of ocular discomfort and dry eye. However the only available direct clinical measure of tear film stability is the TBUT. Studies reporting the association between subjective symptoms of dry eye and objective tests have shown weak associations or less strong clinical associations [562-564]. Because the definition of dry eye includes the triad of ocular surface disease, tear film instability and ocular irritation, it is possible that the weak associations reported between subjective symptoms and objective signs may be due to limitations in the existing methods of measuring qualitative and quantitative discomfort. This raises at least three important questions:

1) What other method can we employ to measure ocular discomfort?
2) Can we bridge the gap between the qualitative (domain sampling) method and quantitative (psychophysical scaling) method of rating discomfort and pain?
3) Is there any correlation between the sensations, ocular surface appearance and the physical characteristics of the stimulus?

The “Comfortscope” model was the result of an attempt to answer these questions and to bridge this gap between the qualitative (domain sampling) and quantitative (psychophysical scaling) methods of recording discomfort and pain caused by tear drying. This is the first study to simultaneously record, discomfort intensity, ocular surface appearance and attributes of sensation. The intensity of ocular discomfort and pain was measured on a 0-10 scale. The 0 in the scale is a meaningful zero point where the subject does not perceive any

discomfort after the opening of the previously closed eye. The upper end of the intensity scale was associated with the physiological blink reflex and when ocular discomfort and pain was intense, reaching 10, the discomfort or pain necessitated blink or closure of the eye. A change in the measure of discomfort was recorded at every 0.2 seconds. The results clearly emphasize that the multidimensional attributes of the pain sensation can be captured simultaneously for a better understanding of discomfort and pain. The "Comfortscope" proved to be a useful tool for rating the various attributes of discomfort and pain related to tear drying.

The discomfort intensity:

In the majority of the subjects the ocular sensation before blink consisted of three phases. Upon opening the eye there was a brief period with no change in the nature of the sensation. The blink mechanism therefore not only reestablishes the tear film but it also resets the altered state of comfort caused by tear breakup. In videotopographic measurements of the anterior tear film and the central cornea it was shown that the corneal surface became more regular in the first few seconds after the blink [565]. Although the direct influence of this surface regularity upon discomfort is not known, it seems that upon blinking the level of ocular discomfort is returned to a "basal" state and remains at this level during minimal or no alterations on the ocular surface. An up-regulation of this basal state may result in the manifestation of the persistent symptoms of discomfort seen in dry eye disease.

The second phase was due to a slowly rising phase of discomfort and the slope of this phase was less steep compared to the slope of the third phase in 67.7% of the subjects. As evidenced by the power functions noted in the results, this second phase has complex associations with tear drying. At present the different models of tear breakup and subsequent drying caused by initial occurrences such as lipid contamination of mucous layer or a mucous rupture of the tear film do not clearly explain the events underlying the psychophysical ratings [448, 452, 454]. But in the majority of the subjects the short TBUT evoked a response from the ocular surface that resulted in a rapid increase in discomfort and was seen as a steep slope. A long TBUT caused a more gradual increase in discomfort and this was seen as a less steep slope. These negative correlations between the TBUT and the slopes suggest that the steep slope of rising discomfort is to a certain extent driven by a

rapidly increasing instability or mechanical disruption of the tear film causing the rapid recruitment of nerve fibers (therefore more steep slopes) and less rapid mechanical events seen in a long TBUT recruit the nerve fibers less rapidly (hence less steep slopes). But tear break up is associated with more complex neurochemical mechanisms and this simple account is incomplete to explain this complex event.

The functions used to describe the intensity data determined a "variable elbow" position and the correlation between the TBUT and the elbow indicated that the shorter TBUT resulted in a quicker rapid onset of discomfort. It is not surprising that the TBUT and elbow are strongly associated because blinking is to a certain extent driven by the ocular surface stimuli and would normally seem to be occurring in the beginning of the third phase.

The third phase of rapidly rising discomfort was characterized by a more steep slope. This steep slope results from subjects forcing their eyes to be kept open. The act of forcible opening of the eye causes a rapid increase of discomfort and signals the need for the eye to blink. These results are similar to the results of experiment 1 shown in the plot of "Discomfort vs. Tear breakup" (fig 7-1) where the tear breakup preceded the discomfort. In addition there is a poor correlation between TBUT and the stage when the slowly rising discomfort phase begins ($r = 0.1$ in table 1). This would suggest that what drives blinking is not the change from a "no discomfort" state to "slowly rising discomfort" but rather the more rapid transition to rapid discomfort from a state of "slowly rising" discomfort phase. The results also show that the two slopes of phase 2 and phase 3 are positively correlated and when considered in association with the negative correlations of the TBUT, it is possible that "comfortable" people have both phases with less steep slopes (Experiment 2-figure 13) while uncomfortable people have steeper slopes.

In about 17% of the subjects an atypical triphasic pattern of discomfort consisted of a phase of "no change" followed by a phase of "rapid increase in discomfort" and a subsequent "slow phase". This rapid rise of discomfort in the second phase is similar to a steep second phase seen in the "typical pattern" of discomfort. Because nociceptive systems have a low gain it is likely that this initial rapid rise in discomfort is perceived from the presence of factors which signal an impending nociceptive threat to the "tear film/ocular surface function unit". This leads to a rapid recruitment of protective response mechanisms such as

reflex tearing and increased mucin secretion by the conjunctival and corneal epithelium. This sudden increase in tear volume and mucin glycoproteins alters the tear rheology, aids in stabilizing the mechanical tear disruptions and also clears away any chemicals released into the tears. These physiological mechanisms are possibly reflected in the less steep third phase.

Relationship of tear drying to ocular discomfort:

The blink reflex and the associated reestablishment of tear film on the ocular surface returns the eye to a state of "basal comfort". The results of sensation (discomfort intensity response) in relation to stimulus (tear drying monitored by the image) illustrate in a straightforward way (figure 8-16 and 8-17) that discomfort and tear film disruption are highly associated within individuals. The psychophysical ratings of discomfort often closely mirror the tear disruption patterns. An initial rapid tear disruption and drying is often associated with the atypical discomfort pattern characterized by a more steep second phase of discomfort. In previous studies modeling the tear rupture similar patterns of tear film disruptions were termed as linear or exponential [566]. Other studies have reported associations of tear film breakup and ocular sensation [554]. But the simultaneous psychophysical ratings of discomfort along with an analysis of tear drying have not been reported before. In addition to this a more general perspective of the association between tear drying and ocular sensation (figure 8-19) reveals that they are associated within the group. The figure 8-19 also indicates that the tear film changes precede the sensory changes. At present it is not clear what type of the change occurs during disruption, drying and evaporation of the tear film. Hyperosmolarity of the tear film and an alteration in the transmembrane mucin gel layer and a collapse of the hydrophilic mucous of the ocular surface have been postulated as common events. The high correlation between drying and ratings of discomfort ($r = 0.91$) indicates that the alterations in tear film can be immediately perceived and rated by the subjects.

Symptom characteristics:

The visualization of symptom characteristics over time enabled the symptoms to be classified into the three major groups. A mechanical group of symptoms, chemical group of symptoms and itch symptoms. These symptom groups correlate with the functional neurons present on the ocular surface such as the mechano-sensory neurons, C-polymodal with

response to mechanical and chemical stimuli and chemosensory neurons insensitive to mechanical and thermal stimuli. At present there is no documentation about itch fibers in the eye, but as mentioned before there is an increasing body of evidence that a distinct set of fibers encode the itch sensation. The grouping of symptoms into these three classes also strengthens the present tentative correlations existing between the functional type of ocular units recruiting different modalities of stimuli and corresponding sensations experienced by humans. The existing algorithm for ocular discomfort classifies the symptoms mainly into non-tear related and tear related problems [552]. It seems appropriate to classify the symptoms on the basis of the underlying types of functional nerves. Such a classification would enable interventions to address the symptoms on the basis of physiology irrespective of the underlying pathology. Analysis of the symptom characteristics revealed the changing nature of characteristics over the course of the interblink interval as seen in the pie charts in figure 22 and figure 23. During the beginning of the interblink interval the three groups of symptoms reported in decreasing order of frequency were mechanical (including scratchiness and dry sensation), chemical (including stinging and burning) and itch respectively. However just before the blink only two groups of symptoms were reported. These were chemical symptoms and mechanical symptoms in decreasing order of frequency. The chemical symptoms of stinging and burning comprised 91% of the responses. These findings are similar to other reports where forced eye opening reported symptoms of stinging and burning most frequently [554]. The results indicate that the symptom ‘itch’ was commonly reported as an initial symptom while it never immediately precedes the blink. This is also illustrated in figures 8-25 and 8-26 in which it is shown that the initial reports and the initial intensities are different with itch being less intense and earlier than stinging and burning and mechanical symptoms (e.g. scratchiness and dryness). Stinging and Burning are uncommon immediately after a blink but are the commonest symptoms (related to a high intensity) immediately before the subjects blink. The reports of mechanical symptoms were located in between itch and burning sensations. The results clearly established that itch is a symptom characteristic associated with a low value of discomfort intensity during forced eye opening (figure 8-24). However the monitoring of this symptom has tremendous importance against the backdrop of the more common and often disabling condition of pruritis. In experiments relating to the microneurography of C-units, the small axon diameter fibers with

slow conduction velocities were identified as histamine sensitive and responsible for itch. It is possible that similar C-fibers in the eye remain to be identified.

The symptom of dry eye:

A fundamental question that we began with was "Does the eye feel dry?" and whether dryness is a sensation. It is likely that these various pathways interact and produce a complex response. In the skin the dimensions of texture and form perception encode roughness. At present identified in every living cell are the commonly found stretch activated and less commonly found stretch inactivated mechano sensitive channels. The phenomenon of mechanosensation could be due to liberation of extracellular ligands, the presence of the DEG/ENaC proteins residing in the cells and the nerve endings or stimulus conveyed through the gap junctions and intercellular adhesions with the presence of the mechanosensitive enzymes such as phospholipase C and A. Apart from these the integrin receptors which mediate cell to cell adhesion play a role in transducing physical forces. In the eye following mechanical stresses there is an increase in the levels of (TGF)-beta2, integrin beta1 and tenascin (TN) [101]. It is therefore possible to infer that just as the perception of dryness in the mouth is independent of any sliding frictional force a similar perception of dryness independent of the lid and ocular surface friction is encoded in the eye and this complex phenomenon reported as the symptom of dryness, is conveyed by not only a sensory neuronal mechanism but also by the non-neuronal cells on the ocular surface.

Conclusion:

In conclusion this is the first report of the simultaneous measures of the various aspects of ocular discomfort and pain. The technique provided us novel information about the development of discomfort during ocular surface drying. It also enabled the quantification of the sequence of events preceding blink and demonstrated the strong associations between tear film characteristics (TBUT and tear drying dynamics) and the accompanying discomfort. It also demonstrated that symptom characteristics that accompany tear drying reflect different components of ocular surface sensitivity.

A prominent difficulty encountered was that the image analysis reporting tear drying was a relative measure of drying assuming that the eye upon first opening was at some basal state

of hydration. This assumption was made as the subjects were young individuals with no symptoms of dry eye. Irregular illumination over the region of the ocular surface often caused difficulties and the illumination itself though constant throughout the course of the experiment did not provide optically calibrated images. The discomfort itself was provoked by forced eye opening and it is essential to study the phases of discomfort during the normal interblink interval by employing non-invasive TBUT methods.

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