Aspects of
Corneal Fluorescein Staining

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

I would like to acknowledge the names of my co-authors who contributed to the manuscripts contained in this thesis:

- Desmond Fonn MOptom
- Natalie Hutchings PhD, MCOptom
- Lyndon Jones PhD, DSc, FCOptom
- Sruthi Srinivasan PhD, BSOptom
- Jalaiah Varikooty MSc

Abstract

PURPOSE

Evaluating the cornea for epithelial fluorescein staining is a key element of the ocular examination of contact lens wearers and people with dry eye disease. It has long been viewed as a method of visualizing a break in the protective epithelial layer, the integrity of which is regarded as vital for protecting the eye and maintaining good vision.

There has been very little reported on the typical staining presentation in dry eye. Understanding more about the distribution of epithelial staining in dry eye disease would be valuable to guide evaluation of a treatment's physiological efficacy. This thesis aimed to determine whether the corneal staining of subjects with symptomatic dry eye presents in a specific distribution pattern

Since 2002, the epithelial staining phenomenon of solution induced corneal staining (SICS) has been investigated. The cause of this staining has been suggested to be due to molecular adhesion rather than physiological damage, but the current evidence is equivocal. More investigation of this phenomenon is warranted to understand the process and the clinical significance of SICS. This thesis aimed to investigate the type, severity and pattern of staining that occurs in SICS, and assess the impact on epithelial cells using *in-vivo* confocal imaging.

METHODS

Chapter 2 described the CORE corneal staining scale., which uniquely reports the *type* and *extent* of the corneal staining on a scale of 0-100. This was the staining scale used to record the level of solution induced corneal staining in all the clinical trials featured in Chapters 5 and 6.

Chapter 3 reported an experiment which was conducted to assess the agreement among fifteen observers who used this scale, in two grading sessions, to grade the corneal staining illustrated in 22 photographic images. Inter- and intra-observer agreement results were calculated.

Chapter 4 presented a meta-analysis of the corneal staining observed in 368 subjects, across 13 studies, with symptoms of dry eye. For each subject the corneal zone of worst staining was recorded to analyse which region of the cornea most frequently exhibited the most severe staining.

In Chapter 5, 20 subjects were exposed to a lens/solution combination, known to induce SICS, in both eyes for a two hour period. In phase one, one lens was rinsed thoroughly before being worn; in

phase two, the eye itself was rinsed thoroughly post lens wear; in phase three, confocal microscopy was conducted on both eyes to look for hyper-reflective epithelial cells. In phases one and two, the epithelium was assessed for staining pre and post lens wear with and without fluorescein.

Chapter 6 evaluated aspects of the staining data collected in several SICS-inducing studies. The frequency of the reported 'donut' pattern of staining was calculated, relative to a diffuse, pan-corneal staining pattern. Seven subjects were identified that had participated in three or more trials using the same SICS-inducing methodology. The data from these individuals were assessed to determine the repeatability of the level of induced corneal staining in these trials.

RESULTS

The CORE corneal staining scale agreement experiment, in Chapter 3, supported the benefit of training because the concordance of the naïve observer was markedly worse than the observers who had received prior training. The inter- and intra-observer agreement analyses provided valuable data which can be applied to the development a pictorial reference guide and better instructions.

The Chapter 4 meta-analysis of the geographic distribution of corneal staining among subjects with symptomatic dry eye demonstrated that the greatest degree of staining was most frequently in the inferior zone. (52.5%) The zone affected least was determined to be the central zone (12.8%).

In the SICS experiment of Chapter 5, rinsing the lens prior to wear and rinsing the eye post lens wear did not result in different staining to the non-rinsed condition. All eyes, irrespective of any rinsing treatment, presented with punctate staining over >84% corneal area. The SICS staining was visible before fluorescein was instilled as 'white light staining'. Confocal images were obtained from 34 of the 40 eyes, and hyper-reflective cells were visible in 33 of those 34 eyes.

The meta-analysis in Chapter 6 concluded that the 'donut-ring' staining pattern, which is often described as typical of SICS, was actually far less common than a diffuse pan-corneal staining presentation. When SICS responding eyes were defined as exhibiting staining of ≥10% extent in at least four of the five corneal zones, 89% were identified as presenting with the pan-corneal pattern i.e. all five zones met the ≥10% extent criteria. When the SICS definition was tightened to include only those with ≥50% extent in at least four zones, 76% of subjects still identified as the pan-corneal staining pattern. There was minimal evidence of SICS presenting with a repeatable degree of staining in the same individual across different clinical trials.

CONCLUSIONS

This thesis investigated several aspects of corneal epithelial fluorescein staining and the chapters have furthered understanding in this field in several ways.

The CORE corneal staining scale provides valuable data regarding the percentage of the corneal affected by staining. The results of the Chapter 3 agreement experiment provide useful information for the next steps in the development of this scale which will create a valuable corneal staining assessment tool.

The evidence that the most severe corneal staining in patients with symptoms of dry eye most often presents in the inferior zone is invaluable to the design of future clinical trials of dry eye treatments. It highlights the importance of specifically assessing this region and the value in targeting fluorescein staining improvements in this zone as a key outcome measure.

SICS has been suggested to be due to adhesion between PHMB (or other care system components) and the epithelial cells. The experiment in Chapter 5 confirmed that rinsing the lens does not remove enough PHMB from the lens to prevent SICS, and rinsing the eye afterwards is not effective at removing the bound molecules from the epithelial cells because SICS is still evident post rinsing. The presence of 'white light staining' and hyper-reflective cells on *in-vivo* confocal microscopy indicate that there are changes to the epithelial cells even before the fluorescein is instilled into the eye. More investigation of changes at the cellular level are required to understand what is happening.

The meta-analysis of SICS data was able to provide evidence that SICS most commonly presents as a diffuse punctate staining that affects the entire cornea presenting in a pan-corneal pattern, rather than presenting in the commonly described pattern of a donut-ring, which implies central zone sparing. The examination of SICS in seven subjects across several studies questions the repeatability of the SICS phenomenon. A targeted repeatability trial is required to conclusively answer this question.

Acknowledgements

I would like to offer a special thank you to my supervisor, Dr. Lyndon Jones. You gave me the confidence to begin this journey and you helped me find the strength to continue through difficult times. Thank you for always making time for me and for your constant encouragement, guidance and enthusiasm. I am grateful for every opportunity you have offered me, and I am very proud to work alongside you.

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I appreciate the assistance I received from the Graduate Coordinators; Krista Parsons, Lisa Baxter, Jennifer Cosentino, Stephanie Forsyth and Holly Forsyth. I also thank the Graduate Officers for their counsel; Drs. Vivian Choh, Trefford Simpson, Daphne McCulloch, Paul Murphy and, particularly, Dr. Benjamin Thompson.

Finally, I am indebted to the many friends and colleagues who provided assistance along the way; personal, professional and academic. There are too many to list by name, but three people deserve specific mention. Debbie Jones and Kathy Dumbleton, you are two very special friends, who supported and encouraged me to continue this work. Daryl Torgrimson, your thoughtfulness, care and understanding kept me fed, watered and smiling to the end.

Dedication

To my parents, Mary and Jim Griffiths.

You taught me to believe in myself. I miss you both.

To my 'boys', Josh and Sam.

You are my inspiration and my strength. I love you both.

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List of Abbreviations

ANOVA	Analysis of variance
CCC	Correlation coefficient of concordance
CCLR	Centre for Contact Lens Research
CORE	Centre for Ocular Research & Education
DE	Dry eye
DEWS II	Dry Eye Workshop II
FDA	Food and Drug Administration
HR	Hyper-reflective
MPS	Multipurpose solution
OSDI	Ocular Surface Disease Index
PATH	Preservative associated transient hyperfluorescence
PHMB	polyhexamethylene biguanide
SD	Standard deviation
SICS	Solution induced corneal staining
SiHy	Silicon hydrogel
TFOS	Tear Film and Ocular Surface
Wk	Week

Chapter 1
Background and Literature Search

The eye is a complex matrix of many different tissues, structures and systems. A failure in one tissue or structure has potential for serious impact on the eye's primary function – vision. The cornea is the most anterior structure of the eye and it is exposed the external environment, protected only by the tear film and the eyelids. The cornea is composed of five layers and the outermost layer is the corneal epithelial layer, Figure 1-1. Sodium fluorescein is a liquid dye that has been routinely used in clinical practice for over fifty years to assess the health of the ocular surface, particularly the corneal epithelium.[1, 2] This thesis explores aspects of sodium fluorescein staining of the corneal epithelium in the presence of symptomatic dry eye and in the presence of solution induced corneal staining (SICS).

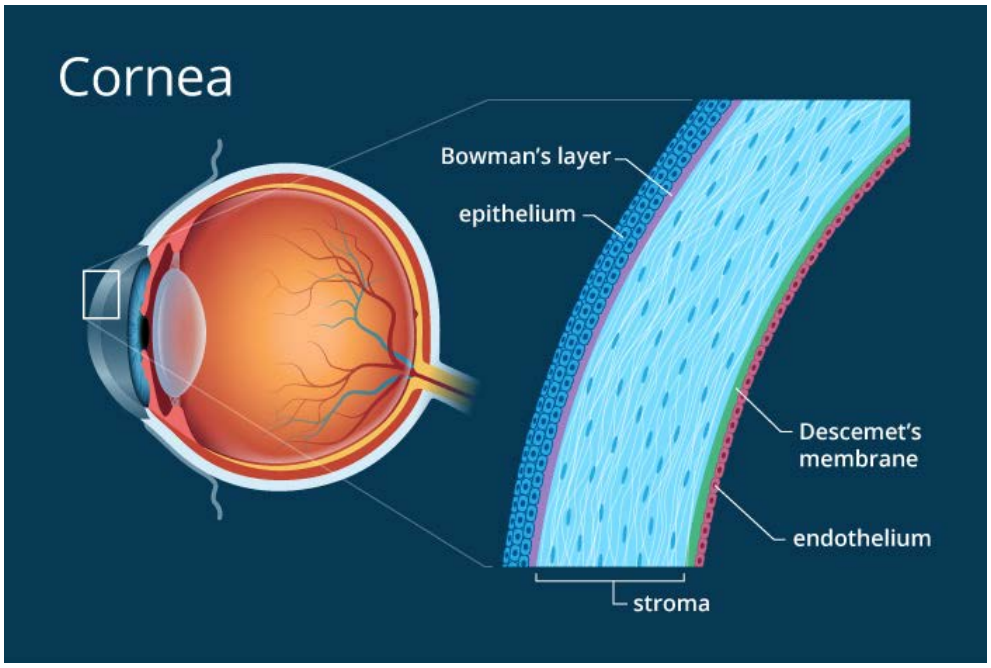

Figure 1-1: Schematic showing the five corneal layers. Image reproduced from: https://www.allaboutvision.com/resources/cornea.htm.[3]

1.1 Corneal structure and relationships

The cornea is the anterior face of the eye, which acts as one of several 'windows' through which vision is possible. Because light passes through the cornea on the way to the retina, it is vital to visual function that the cornea remains as optically clear and free from distortion as possible. The requirement for clarity means the cornea is avascular. It draws nutrients from its surrounding environments; tear film, aqueous humour, limbal blood vessels and inner tarsal blood vessels.

From the frontal view, the cornea appears as a round disc of clear tissue, situated directly in front of the iris and pupil, which meets the sclera and conjunctiva at its circumference, a junction called the limbus. From the lateral perspective, the cornea appears as a dome structure of smaller radius of curvature than the rest of the eyeball. Remaining distortion free while maintaining a vertically oriented, curved shape requires a robust structure. The cornea is approximately 535μm thick at the centre, slightly thicker at the periphery and is composed of five distinct layers.[4, 5] The corneal curvature is largely maintained by the lamellar structure within the thickest corneal layer, the stroma.[6]

The five corneal layers are shown in Figure 1-2. The most anterior surface of the cornea is the corneal epithelium, which is exposed to the tear film and the external environment. Next is Bowman's membrane, which is also known as the anterior limiting membrane. The stroma is the middle layer and makes up the vast majority of the corneal thickness. Posterior to the stroma is the posterior limiting layer, Descemet's membrane. Lastly, the innermost layer is a single cell layer, the endothelium.

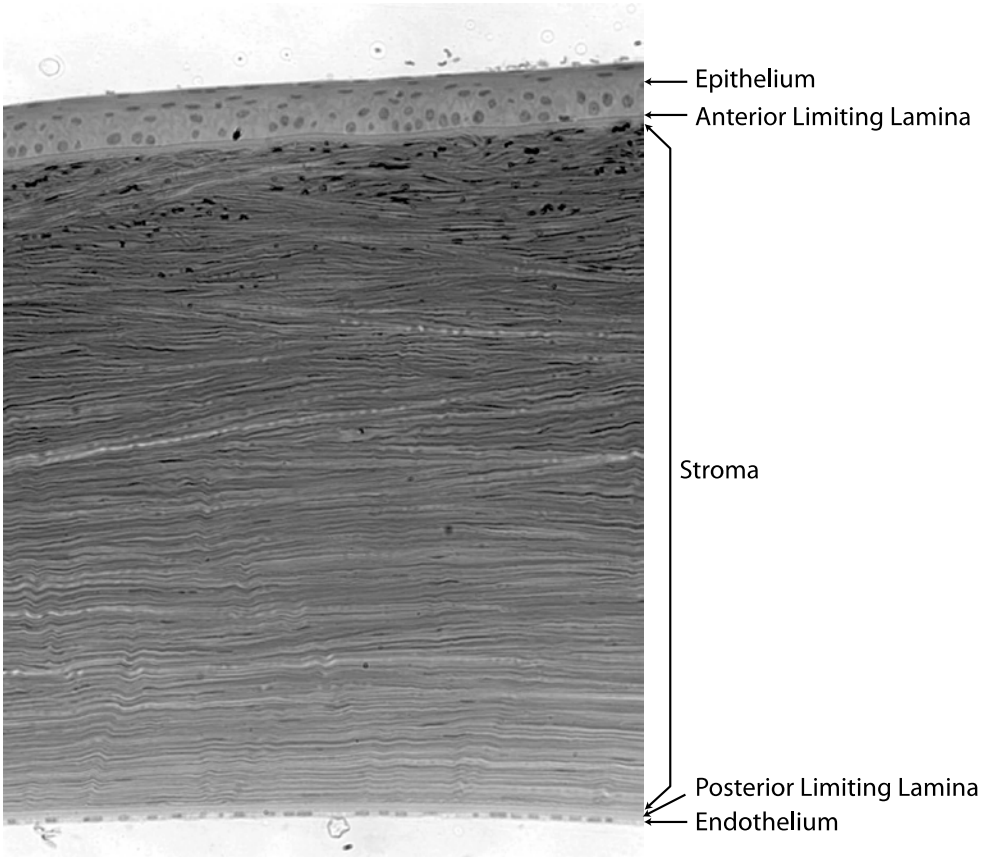

Figure 1-2: Corneal layers of a primate cornea. Electron micrograph, magnification x100. Image courtesy of Jan P. G. Bergmanson.[7]

1.1.1 Epithelium

The epithelium is the outermost layer of the cornea. It is immediately adjacent to the tear film and is the external protective layer for the cornea and therefore the eye. The corneal epithelium is approximately 51μm thick at the centre of the cornea and thicker at the limbus, where it is continuous with the conjunctival epithelium.[5] It is made up of 5-7 layers of nucleated, non-keratinised, epithelial cells layered according to the three stages of their seven day life cycle; basal, wing and squamous.[8] As the cells age, they migrate towards the anterior surface where they eventually slough off into the tear film.

There are difficulties obtaining representative images of human epithelial cells before they degrade, however the three cell stages can be seen in Figure 1-3, which is a primate cornea and in the light microscopy of a human cornea, Figure 1-4, which also shows the anterior stroma.

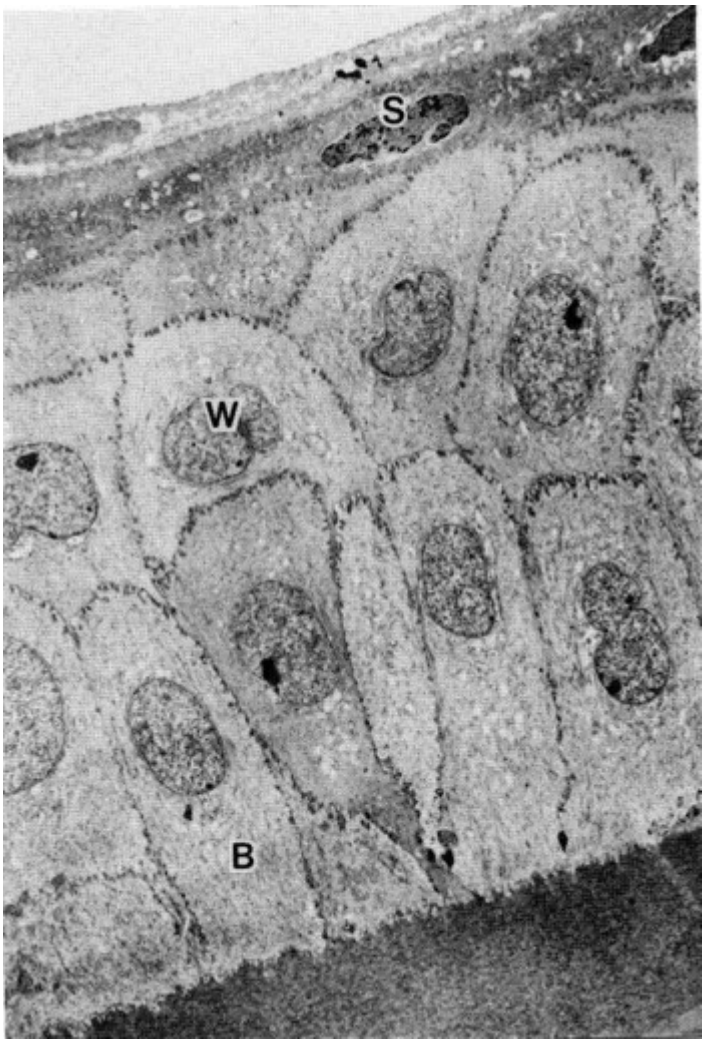

Figure 1-3: Epithelium of a primate, illustrating the epithelial layers from the columnar, basal cells (B), to wing cells (W) ending with the flatter, anterior surface, squamous cells (S). Electron micrograph, magnification x11,000. Image courtesy of Jan P. G. Bergmanson.[7]

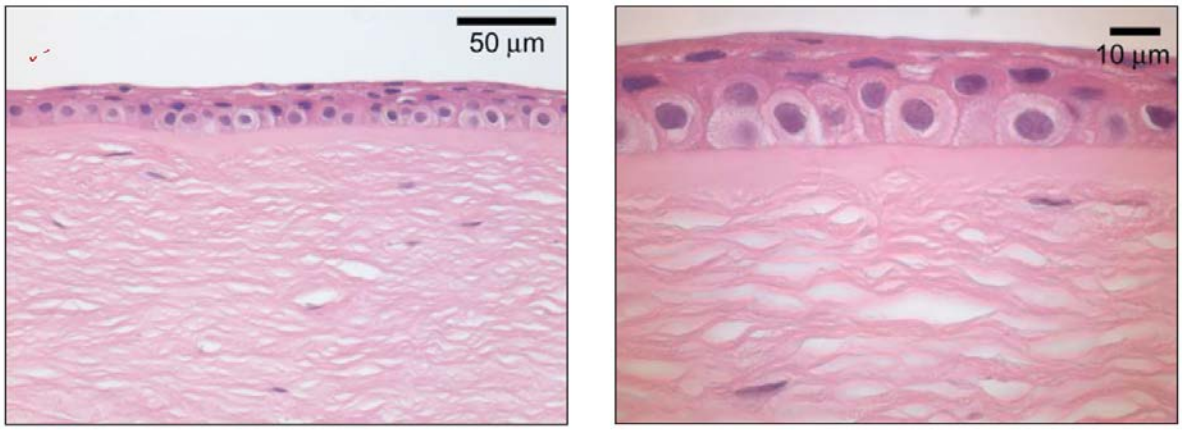

Figure 1-4: Human epithelium and anterior stroma. Light microscopy, magnification x400. Image reproduced from: Becker U, Ehrhardt C, Schneider M, Muys L, Gross D, Eschmann K, Schaefer UF, Lehr CM. A comparative evaluation of corneal epithelial cell cultures for assessing ocular permeability. Alternatives to laboratory animals. 2008. 36(1):33-44.[9]

The cells of the apical (anterior) layer are often referred to as squamous cells. They are somewhat flattened and lie parallel to the surface. These outermost cells are the oldest and they will slough into the tear film as they are replaced from below. The outer-facing membranes of these apical epithelial cells are covered with an irregular network of microvilli, believed to be fundamental to tear film adhesion and tear film stability.[5] These outer-facing membranes secrete a glycocalyx, or film, which serves as an additional barrier and hinders the binding of organisms to the epithelial surface. Recent work has highlighted the interactions of three mucins, MUC1, MUC4 and MUC16, with the protein galectin-3 as a significant component of this protective layer.[10] Within this layer, tight-junctions are present between all adjacent epithelial cells, creating an impermeable barrier to the diffusion of pathogens and molecules into the intercellular space.[10] Desmosomes (anchoring junctions "binding" adjacent cells together) and adherens-junctions in the various layers of the corneal epithelium provide structural integrity and further prevent fluid from the tear film overhydrating the cornea.[10] Cell junctions play a key role in corneal integrity.[10]

The deepest epithelial layer, also referred to as the basal layer, is comprised of a single layer of cells which are columnar in shape and oriented with their long axis perpendicular to the corneal surface. These cells secrete the basement membrane to which they are strongly bonded via hemi-desmosomes. As new cells are produced in this basal layer, the older cells migrate forward and

change orientation, forming 2-3 layers of wing cells immediately below the squamous rows.[5] In the wing and basal cells layers there are no tight-junctions, and therefore some fluid can move from the stroma into the epithelium.

The epithelial layer of the cornea is constantly regenerating, yet in spite of this constant cellular movement to the surface, the cell boundaries are extremely strong and the epithelium remains firmly anchored to the epithelial basement membrane, which is immediately adjacent to Bowman's membrane. In corneal homeostasis, the replenishment of epithelial cells in this continual shed/renewal cycle assists in the maintenance of a uniform structure which, in turn, helps maintain corneal transparency.[11] The continual replacement of lost cells is possible because of the presence and activity of stem cells which are found within the basal epithelial layer, mainly at the conjunctival border, the limbus; specifically in the palisades of Vogt, epithelial crypts and stromal projections.[12, 13] These stem cells, as in other self-regenerating tissues within the body, are capable of unlimited self-renewal.[14] The largest stem cell reservoirs are found at the superior and inferior limbal regions, however the annular distribution provides the entire cornea with a constant supply of new cells which migrate centrally from this outer annulus.[15] Larger corneal epithelial wounds have been shown to heal from the outer edges inwards, with faster healing in the peripheral cornea than the central, validating the centripetal movement of the stem cells from the limbus.[16, 17] The annular stem cell population also acts as a barrier to conjunctival cells which may otherwise migrate into the cornea causing a loss of corneal transparency.[18] It is clear that the role of these stem cells is vital to corneal, and therefore ocular, integrity and thus damage to the peripheral epithelium should always be taken seriously.

1.1.2 Bowman's membrane

Bowman's membrane is an acellular membrane that separates the stroma from the corneal epithelium. It is 8-10μm thick and attached strongly to the anterior stromal lamellar.[19] It functions as a protective layer for the stroma, however once damaged it cannot be repaired and any extensive damage will likely result in permanent scarring.[1]

1.1.3 Stroma

The stroma makes up 90% of the corneal thickness and is organized as a matrix of collagen fibril lamellae with scattered large flat, paper-thin keratocytes.[5] The density of the keratocytes reduces in the posterior stroma, where the collagen lamellae become thinner.[20] The keratocytes maintain the

collagen fibrils.[19] The strong structural architecture of the stroma maintains corneal shape and acts as a barrier to physical and chemical injury.

1.1.4 Descemet's membrane

This is an acellular layer which acts as the basement layer for the endothelial cells, separating the endothelium from the stroma.

1.1.5 Endothelium

The endothelium is the innermost layer of the cornea. Just 5μm thick, it is comprised of a single layer of squamous cells which do not replicate or reproduce. This monolayer functions as the pump which maintains optimal corneal hydration by moving fluid out of the cornea via complex ion transport systems.[21] This pumping role is vital in maintaining corneal clarity, as excess fluid buildup causes clouding of the cornea, called oedema. The endothelium cells are adjacent to the aqueous humour and therefore the pump mechanism stops the aqueous humour from flooding through the endothelium causing corneal oedema. Throughout life, endothelial cells are gradually lost, causing neighbouring cells to stretch to fill the void.[21] Thus, the cells of this layer become increasingly polymorphous with age, Figure 1-5.[21, 22]

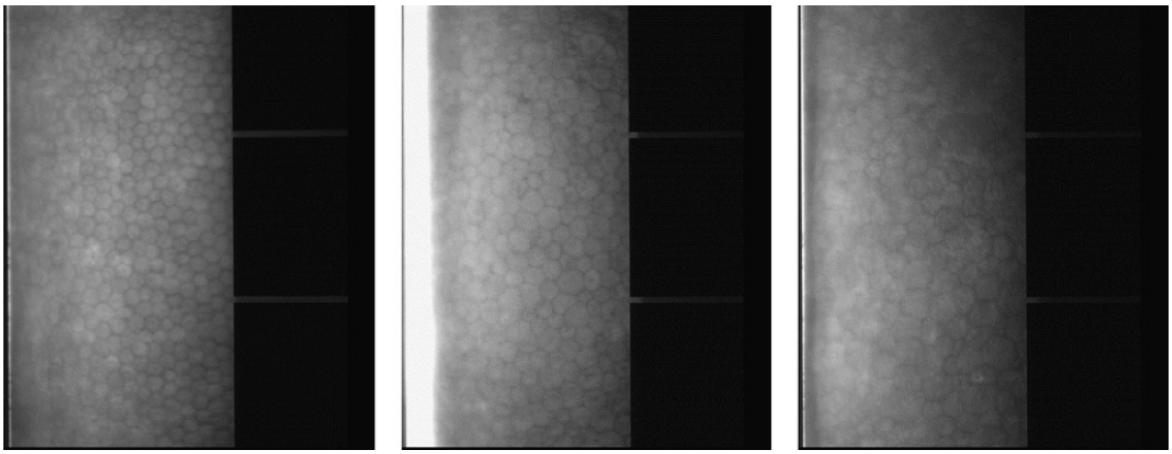

Figure 1-5: Representative images of the corneal endothelial mosaic appearance changes with age. Image reproduced from: Rannou K, Crouzet E, Ronin C, Guerrero P, Thuret G, Gain P, et al. Comparison of corneal endothelial mosaic according to the age: The CorImMo 3D Project. IRBM. 2016;37:124-30.[22]

1.2 Corneal innervation

The cornea is a densely innervated structure. Axons that mostly originate from the long ciliary nerves enter the cornea at the level of the anterior stroma or posterior epithelium, at various positions around the limbus. These axons then branch, largely remaining within their layer, thus creating a basal epithelial plexus and a less dense anterior stromal plexus.[5] There are occasional axons passing through Bowman's membrane connecting these two nerve plexi.[4, 5] All the nerves in the epithelium lose their protective myelin sheath at the limbus, another structural detail to preserve the optical integrity of the cornea, however, the myelin remains around the nerve fibres within the stroma.[5] Stimuli for corneal nerves include touch, cold, osmolality and pH, but all the responses are perceived psychologically as 'pain', thereby invoking a protective response from the host.[23]

1.3 Assessing corneal integrity

As mentioned earlier, the cornea's integrity is essential to facilitate clear vision and the corneal epithelium is the primary barrier to external pathogens. A method for assessing the corneal epithelium's integrity is thus desirable. There are only a few organisms that can invade an intact epithelium in a healthy person to cause a scar-inducing infection,[24] therefore maintaining tissue integrity is an important part of avoiding infection. While corneal trauma and surgery are obvious risk factors for infection, exposure keratitis (as in dry eye) and contact lens wear have also been recognized as major predisposing factors.[25] Therefore, in individuals with a history of dry eye or contact lens wear, evaluating the integrity of the corneal epithelium is a very important clinical assessment, both for a proactive management approach as well as part of reactive, problem solving management.

The standard equipment for assessing the human cornea *in vivo* is an optical slit-lamp biomicroscope. These microscopes comprise a binocular viewing system coupled with an illumination system such that both systems share a centre of rotation and therefore share focusing and movement systems.[26] The coincident focusing of light and microscope, combined with an easily adjusted magnification, make the slit-lamp biomicroscope a fundamental piece of equipment in optometry and ophthalmology practices which is used to routinely inspect the anterior eye and conduct contact lens follow-up assessments, Figure 1-6.

Slit-lamp biomicroscopes produce a focused beam of light but an optional diffuser can be moved in front of the illumination system as required. Diffuse illumination allows general observation of the surface ocular tissue, tear film and corneal epithelium, best used with low magnification of around 10x or less. A broad, direct (non-diffused) light beam provides a more intense light source to inspect specific structures or layers, typically used with the microscope at the same angle as the light source. A focused beam of 2mm width with the observation microscope set at an angle facilitates a view of the layers of the cornea as the light beam penetrates the structure, creating a parallelepiped. If the beam is narrowed considerably and the magnification is set to 32x or 40x, then an optical section is created, and the five layers of the cornea can be observed. With this setting, any distortions or defects within the structure become apparent. The maximum magnification for a slit-lamp is 40x, allowing resolution as low as 30μm.

Figure 1-6: Slit-lamp biomicroscope. Image courtesy of CORE.

Confocal microscopy affords magnification of around 680x, which permits individual corneal cells to be imaged. It is a contact microscope requiring anaesthetic and a gel interface between the objective lens and the cornea. This method provides images of multiple sections of the cornea which are ‘front-on’ sections of a single layer, rather than sections through all the corneal layers. These

instruments became available in the late 1990s and more information on the theory behind them is summarized elsewhere.[27]

High magnification is also afforded by a specular microscope. However, the principles for its use rely on a refractive index differential and therefore the endothelium is the structure best viewed with this microscope as other corneal layers are too similar in index to their neighbouring layers for good resolution. This makes the specular microscope too limited in application for a practice setting. Specular reflection is attained when the angle of illumination equals the angle of the observation microscope. Using this technique with a slit-lamp allows observation of the gross appearance of the endothelial cells. More detail on specular microscopes can be found elsewhere.[28, 29]

The highest magnification of human tissue is achieved with the electron scanning microscope. However, these microscopes are only applicable to *ex-vivo* or cultured tissue. Such high magnification of live tissue in situ in the body is not yet achievable. Despite this drawback, much detail about the corneal anatomy was learnt using electron microscopy.[5]

Due to the need for optical clarity of the corneal structure, in a healthy cornea there is, quite literally, very little to see. Areas of the cornea can appear opaque due to the presence of anomalies such as scars, foreign bodies and oedema. While the vitality of the entire cornea is important to maintain good vision, the epithelium is of particular importance because of its positioning as the anterior barrier layer, exposed to the external environment. The use of stains has become commonplace to examine the epithelium because they can assist in screening for early signs of damage or aid in diagnosis of disease and/or damage. Sodium fluorescein, rose bengal and lissamine green are the three commonly used stains for examining the integrity of the ocular surface in conjunction with a slit-lamp biomicroscope.[26] Sodium fluorescein is regarded as the most valuable stain to assess corneal integrity[26] and therefore, as per the scope of this thesis, only the interaction of sodium fluorescein with the corneal epithelium will be discussed in more detail below.

1.4 Sodium fluorescein interaction with corneal epithelium

1.4.1 Sodium fluorescein

Fluorescein is a manufactured, organic, soluble compound which is used as a dark orange/red dye in many applications, ranging from water system leak detection to an injectable medical tracer.

The first reported use in the eye was in 1882 by Pfluger.[30] He combined fluorescein with sodium to improve its solubility, and described the view of "corneal staining" when he instilled it into rabbit eyes. It is the sodium fluorescein compound which is the formulation used widely today in the optometry and ophthalmology fields to examine the ocular surface. Throughout this thesis the term fluorescein is used and refers to sodium fluorescein, also known as fluorescein disodium salt.

1.4.2 Properties of sodium fluorescein

Fluorescein has the molecular structure of $C_{20}H_{10}Na_2O_5$, Figure 1-7. It has a molecular weight of 376 daltons and is 50% soluble in water at 15°C, making it more soluble than sodium chloride.[30]

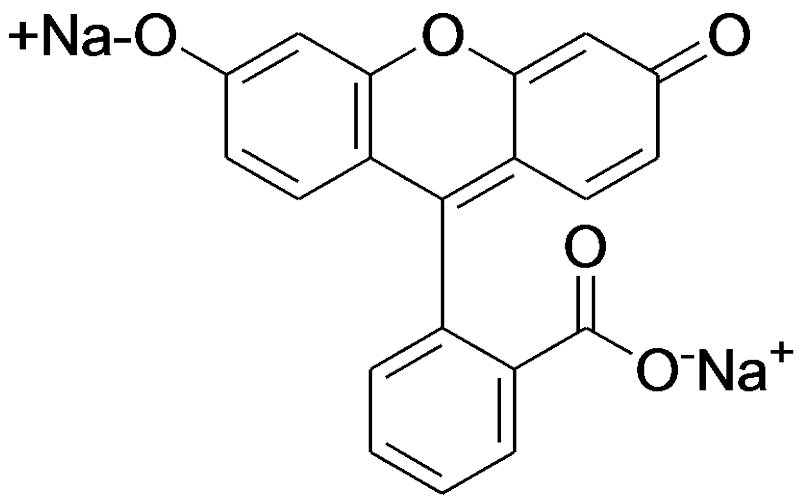

Figure 1-7: Molecular structure of sodium fluorescein.

Fluorescein has many uses in ocular examination. It has been used for over fifty years as an injectable in retinal fluorescein angiography to facilitate examination of the retinal and choroidal circulation.[31] However there are many more ocular uses which are far less invasive and involve instilling fluorescein onto the anterior ocular surface and viewing though a slit-lamp biomicroscope, using filters that cause the fluorescein to fluoresce a bright green colour (see below for more details). In addition to being used to assess corneal epithelial staining, other uses include assessing conjunctival staining, tarsal roughness, ocular injury, corneal epithelial dystrophies, tear film integrity, as well as assessing rigid contact lens fit and facilitating applanation tonometry, Figure 1-8.

All of these uses are possible largely because of the solubility and fluorescent properties of fluorescein, coupled with its low toxicity. While there have been reports of some allergic responses related to intravenous use of fluorescein,[32] the instillation of fluorescein onto the ocular surface is not expected to cause any adverse responses, however repeated instillations have been shown to be associated with increased corneal staining.[33]

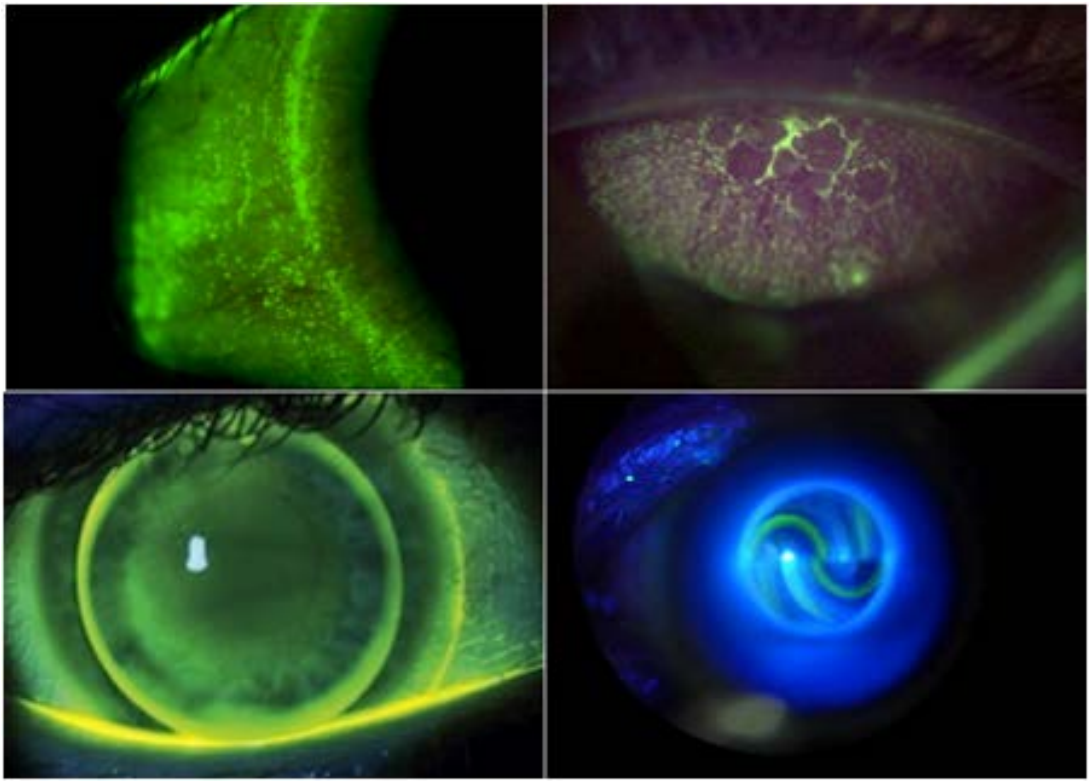

Figure 1-8: Top left: conjunctival staining (image courtesy of CORE); top right: tarsal roughness (image courtesy of Lyndon Jones); bottom left: rigid lens fit (image courtesy of CORE); bottom right: applanation tonometry (image reproduced from https://en.wikipedia.org/wiki/Ocular_tonometry[34]).

It is the fluorescence of fluorescein at very low concentrations that has earned fluorescein its broad ophthalmic use to 'visualize' the tear film and the integrity of the ocular surface.[35] Fluorescein exhibits maximum excitation when exposed to light of wavelength 495nm, Figure 1-9.[36] White light sources do provide this 495nm wavelength however, use of a white light source for this purpose is less than ideal because the emitted fluorescence from the fluorescein is difficult to discern against the

breadth of the background illumination from the broad white light spectra. Thus, observation can be optimised by matching the incident light more closely to that of the maximal fluorescein excitation wavelength, 495nm. This is achieved by placing a blue filter over the slit-lamp light source, often a cobalt blue filter. Many slit-lamps include a blue filter option as standard and ideally this filter would have peak transmittance close to 495nm in order to maximise the excitation of the fluorescein., however Peterson *et al*.[37] have demonstrated that many slit-lamp blue filters have their peak transmittance closer to 450nm, thus offering sub-optimal exitation. Nevertheless, using these blue filters is far more effective than observing with a regular white light source, though their low level transmittance causes difficulties when attempting photography, and other filters have been recommended for this situation.[38]

While an incident light source of 495nm is required for optimal excitation, the wavelength of the fluorescence emitted is around 510-520nm, Figure 1-9.[39] In order to optimise the view of the emitted fluorescence, a barrier filter is recommended which blocks the majority of the available light, leaving only the fluorescence wavelengths visible. A yellow filter, often a Wratten 12 filter, which blocks wavelengths shorter than 500nm, has been traditionally used for this purpose and it is very effective in optimising the view of any fluorescence on the corneal surface by enhancing the contrast. Figure 1-10 illustrates the improved observation that the yellow filter affords when observing epithelial fluorescein staining.

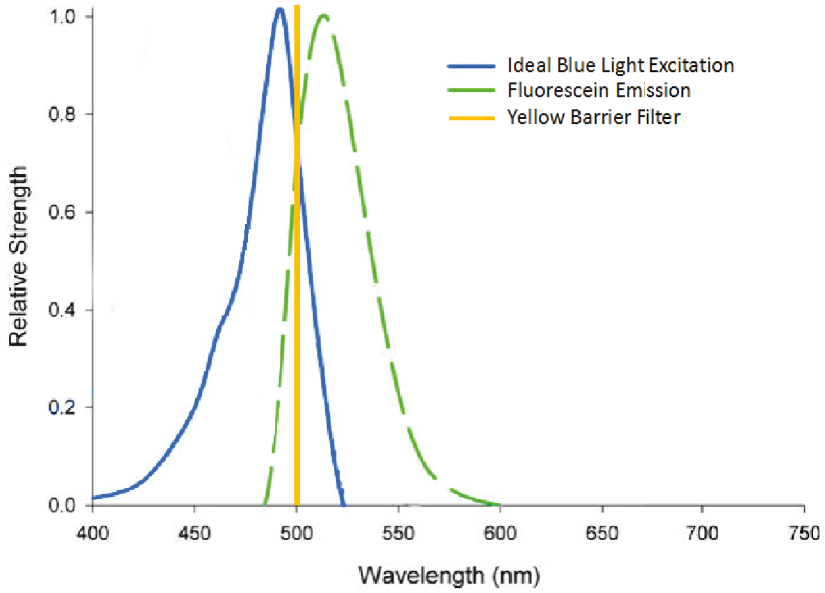

Figure 1-9: Peak excitation and emission spectra of fluorescein; position of yellow barrier filter. Graph adapted from Peterson RC, Wolffsohn JS, Fowler CW. Optimization of anterior eye fluorescein viewing. Am J Ophthalmol. 2006;142:572-5.[37]

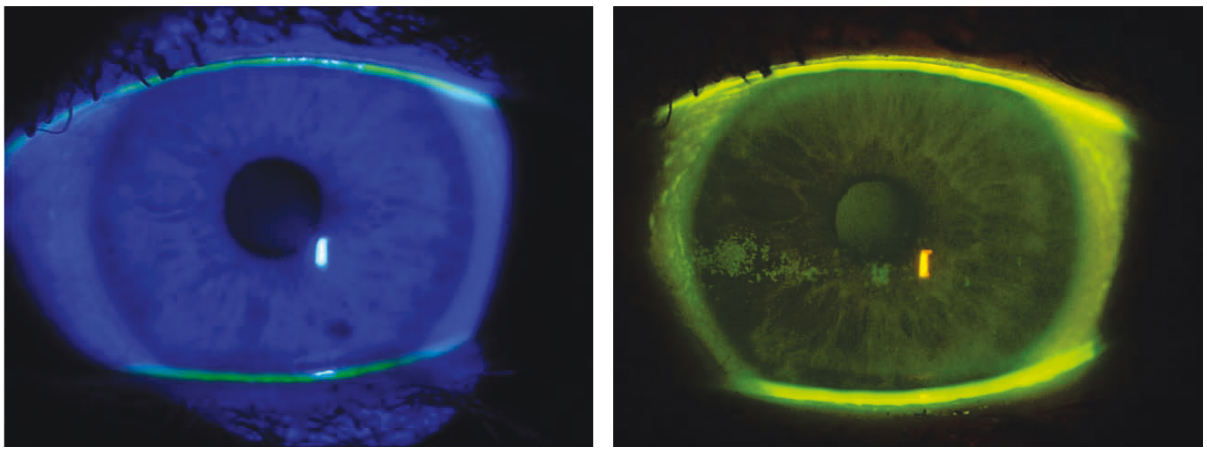

Figure 1-10: Slit-lamp biomicroscope view of epithelial staining with blue filter over illumination: left image with no yellow filter; right image with yellow filter in front of observation system. Images courtesy of L Jones, CORE.

The intensity of the fluorescence of fluorescein is influenced by pH, layer thickness and concentration. The typical pH of the ocular surface is between 6.5 and 8.0 and at this level the light absorption peak is still 495nm and the fluorescence appears the familiar green colour.[35, 36] As the fluorescein stained tear layer becomes thicker, it fluoresces more brightly, a property fundamental to its use in rigid lens fit, Figure 1-8. The fluorescence increases with concentration up to a maximal concentration of approximately 0.001%, with higher concentrations causing a reduction in fluorescence due to a process called 'quenching', a phenomenon in which competitive molecular emission blocks the fluorescence.[30]

1.4.3 Instilling fluorescein into the eye

Because liquid fluorescein has an affinity for contamination with pseudomonas aeruginosa,[40] a bacterium which is highly damaging to the cornea, it is rarely used in the multi-dose 2% concentration liquid form. It is available in unit dose liquid of either 1% or 2% concentrations, but due to the high relative cost of this preparation, in clinical practice fluorescein is most commonly sourced in a single use, dry, paper strip preparation. The small paper strips are impregnated with 0.6-1.0 mg (depending on brand) dry fluorescein compound at one end and each one is individually sealed in a sterile envelope, within a multi-envelope box, Figure 1-11.

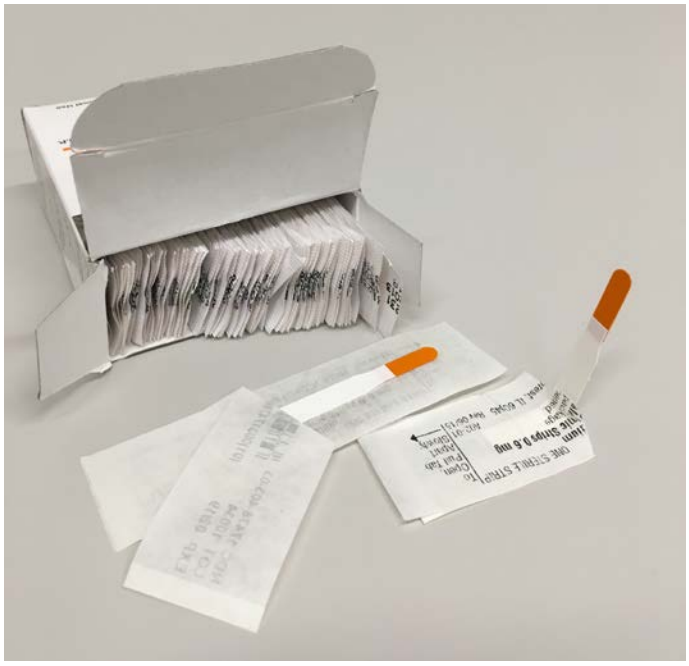

Figure 1-11: Fluorescein strips.

After removing the strip from its envelope, a small volume of liquid fluorescein dye is applied to the ocular surface by wetting the strip with sterile saline and then holding it against the lower lid inner tarsal region, or against the lower bulbar conjunctiva. If not controlled carefully, this method of fluorescein instillation can deliver quite different volumes and concentrations of fluorescein to the eye. CORE uses a standardised fluorescein instillation methodology which was recommended by Peterson *et al*.[37] to maximise the fluorescence observed. This procedure involves placing one drop of saline (approximately 40µl[41]) onto the impregnated end of the fluorescein strip and shaking the strip vigorously to remove excess fluid. The wetted strip is then brought into brief contact with the lower tarsal region, Figure 1-12.

Figure 1-12: Application of a wetted fluorescein strip to the lower tarsus. Image courtesy of CORE.

Abdul-Fattah *et al.*[41] conducted an *in vitro* quantification experiment to estimate the amount of fluorescein delivered by whole and split impregnated fluorescein strips compared to measured volumes of 2% w/v liquid fluorescein. They determined that when a full strip was wetted by 50µl saline, shaken and then applied across 2mm of damp filter paper, it delivered an amount of fluorescein that was equivalent to 3µl of the 2% w/v liquid fluorescein. They also noted that splitting the impregnated strips into thinner strips appeared to offer a simple and effective way of controlling the amount of fluorescein instilled into the eye.

Immediately after initial instillation into the eye the concentration can be higher than optimal and lead to quenching, which would cause under-reporting of ocular surface staining. Peterson *et al*.[37] recommend a wait time of one minute before assessment in order for the concentration to reduce and stabilise within the optimal range, Figure 1-13.

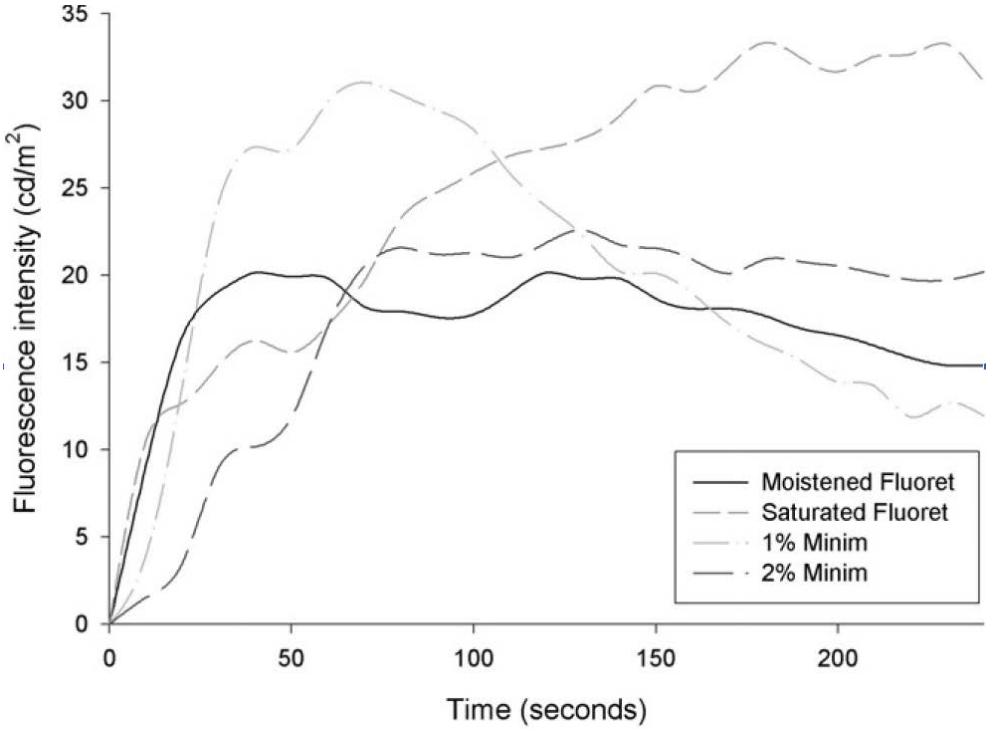

Figure 1-13: Fluorescein average fluorescence intensity profiles, by instillation method. Graph reproduced from Peterson RC, Wolffsohn JS, Fowler CW. Optimization of anterior eye fluorescein viewing. Am J Ophthalmol. 2006;142:572-5.[37]

1.4.4 Viewing corneal fluorescein staining

As mentioned earlier, after instillation of fluorescein into the eye, any staining of the corneal epithelium is optimally observed after one minute, using a slit-lamp biomicroscope with a blue filter over the illumination system and a yellow filter in front of the observation system.[42]

Under these viewing conditions, the fluorescein mixes with the tear film causing it to appear green, which shows as brighter green in the thicker tear meniscus regions along the lid margins, Figure 1-14. If the fluorescein penetrates specific areas of the epithelium, then those areas appear very bright

green, and they are easily discernable against the pale green hue of the tear film, Figure 1-15. These bright green areas are typically termed areas of corneal epithelial staining and, because it has long been believed that fluorescein only enters ocular surface cells if they are damaged,[35] then corneal staining has been presumed to be evidence of mechanical or biochemical disruption of the epithelium cell layers.[35] The significance of corneal staining and the epithelial cell interactions with fluorescein is described in Section 1.8.

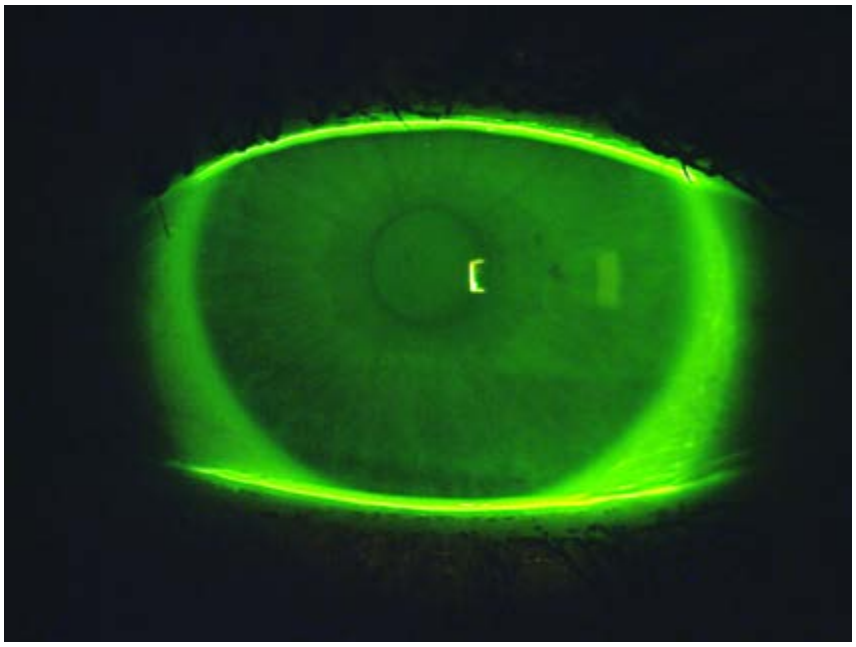

Figure 1-14: Image of ocular surface with fluorescein instilled and viewed with cobalt blue and yellow filters: the tear film appears green.

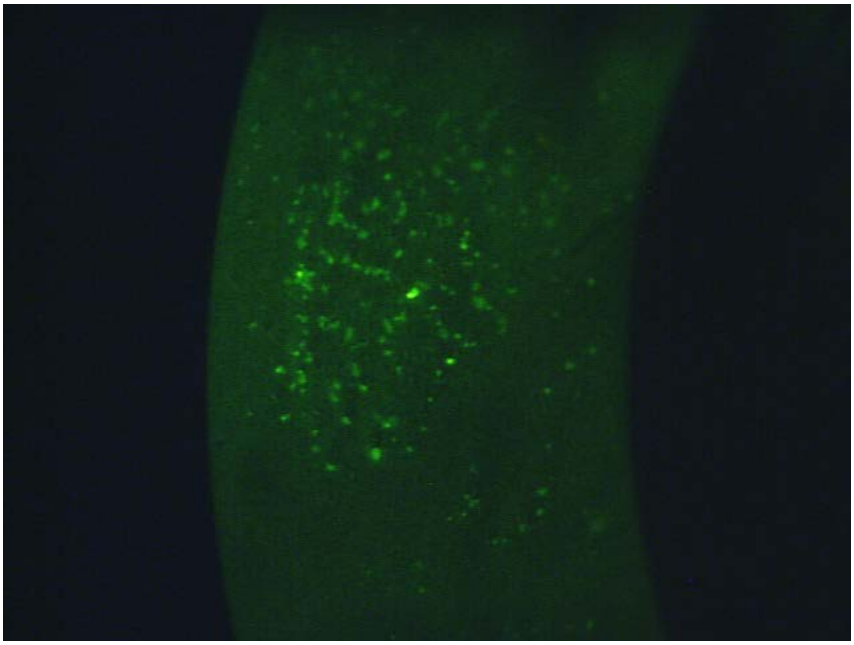

Figure 1-15: Slit-lamp image of cornea, 2mm beam width, 20x magnification, blue light and yellow filter, fluorescein instilled: Bright green epithelial punctate fluorescein staining is visible against the pale green tear film. Image courtesy of CORE.

As described in the previous section, this methodology provides an optimal view of the fluorescein staining because the blue filter transforms the incident light to approximate the peak excitation wavelength of fluorescein, 495nm. This use of blue illumination transforms the orange fluorescein appearance to a bright green, which has greater contrast against the blue incident light than orange against a white light background. The yellow filter in front of the observation system improves contrast still further by blocking most of the blue light, leaving the bright green fluorescein standing out even more defined against a dark background. This observation method is so standard that all slit-lamps provide a blue light option and most have an internal yellow filter too, which is far more convenient than using a separate hand-held one.

1.4.5 Methods of recording fluorescein corneal staining

Because any break in the epithelial barrier provides the opportunity for micro-organisms or other unwanted substances to enter the cornea, potentially causing damage and scarring, assessing and

recording these breaks is important. Recording the degree of epithelial staining provides a quantification of the epithelial insult and facilitates monitoring of worsening or recovery. It has been said that "a picture paints a thousand words", however not all practices have the equipment required to adequately photograph corneal staining. Born from the need to assess the severity and extent of corneal staining to make comparisons over time, several staining grading scales have been developed to describe fluorescein staining.

The early scales were descriptive using terms such as 'trace' or 'severe'. These were followed by numeric scales which hold the advantage of lending themselves to statistical analysis. There are several numeric scales which provide a single numeric grade for the entire corneal surface and of these, some offer integer step scales, such as the Oxford 0-4 scale[43] and the Sjogren's Syndrome International Registry Scale[44] (0-6 scale) whereas the Efron 0-4 scale[45] is designed to be used in 0.1 steps. Other scales recommend to grade the staining of the cornea in five separate corneal zones, the central, superior, inferior, temporal and nasal zones, as first suggested by Josephson and Caffery.[46] Examples of these scales are those of the Food and Drug Administration[47], the National Eye Institute[48], the Brien Holden Vision Institute[49] (formerly the CCLRU scale) and the Centre for Ocular Research & Education[50] (CORE). Chapter 2 describes these in more detail, specifically the CORE corneal staining scale (see Chapter 2 and Woods *et al*.[51]) that was used in the clinical trials described in the following thesis chapters.

Two examples of quite different staining appearances are shown in Figure 1-16. The most common and least concerning form of corneal staining is that of superficial 'punctate' staining, which is visualised as very small dots of bright green fluorescein hyperfluorescence that may be highly localised or widespread across the cornea. It would seem logical that the larger the area of staining, the more the corneal surface is compromised. It is also intuitive to be more concerned about corneal staining which is closer to the visual axis, because any significant surface disturbance at this location could be detrimental to vision. Additionally, the corneal epithelium relies on stem cells to continually regenerate and these are primarily located in the limbal regions, with highest populations at the superior and inferior limbus. Therefore, staining at the limbus, in particular the superior or inferior limbus, would also be of concern.

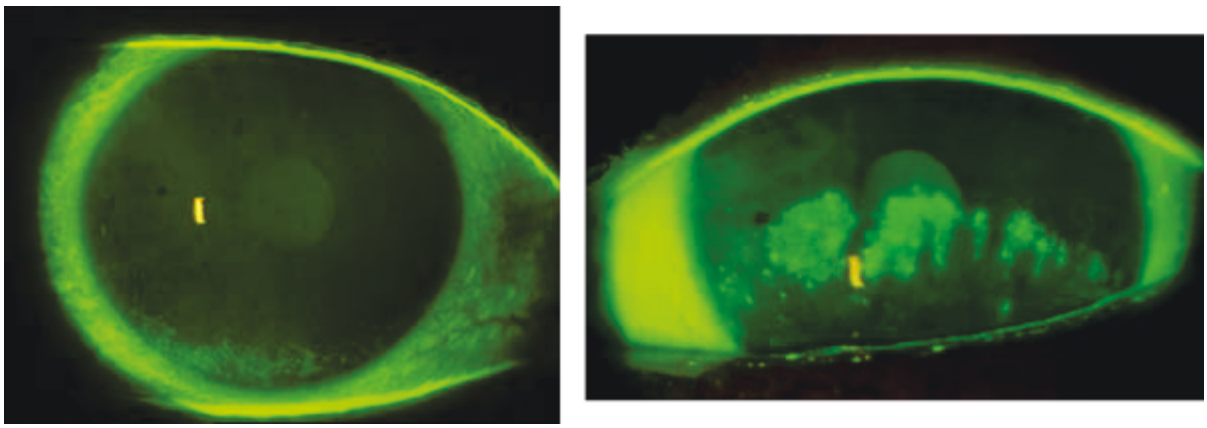

Figure 1-16: Two examples of different staining presentations. Left side: superficial punctate staining limited to the inferior peripheral region. Right side: denser, more coalescent staining that impinges on the visual axis. Images courtesy of CORE.

As the severity of the staining increases then the punctate dots become larger and may eventually begin to merge together or 'coalesce'. A 'patch' stain would be an area that fluoresces completely and does not have the punctate dotted appearance. This would indicate severe epithelial damage and possibly a full thickness break in the epithelium, indicating damage to all the cell layers.

An insult that penetrates the entire epithelium and also compromises or penetrates Bowman's membrane can be recognised because fluorescein will not stay within the epithelium, as in other cases, it will diffuse into the stroma. Fluorescein in the stroma does not present in discretely defined areas like it does in the tightly packed epithelium, but rather spreads between the lamellae presenting as a diffuse, soft-edged, stromal glow, Figure 1-17. The appearance of this stromal glow, along with the speed with which the glow appears, is indicative of the depth and severity of the corneal insult.

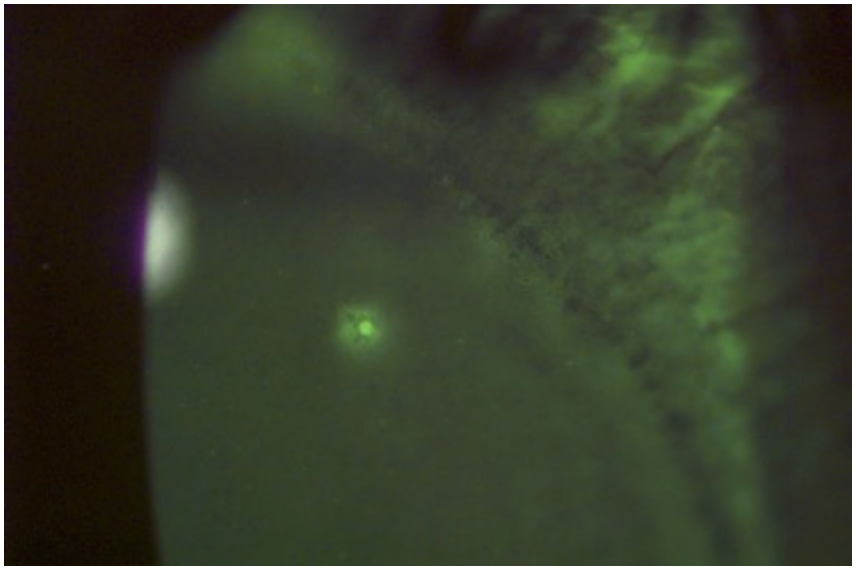

Figure 1-17: Diffuse stromal fluorescein glow surrounding the discrete punctate staining. Image courtesy of CORE.

1.5 Fluorescein staining associated with dry eye

Dry eye disease has been redefined by the Tear Film and Ocular Surface Society (TFOS) Dry Eye Workshop (DEWS) II, Definition and Classification Report[52] as follows:

> *"Dry eye is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."*

There are several symptom gathering methods but the most commonly used, validated questionnaire is the Ocular Surface Disease Index (OSDI) questionnaire, which has been validated[53] and can identify the severity of the dry eye.[4]

This new definition of dry eye disease does not mandate corneal staining to be present to confirm a dry eye diagnosis, however, this Report does state that the degree of staining is an important aspect of severe dry eye disease. Chalmers *et al.*[55] have specifically reported that the presence of >grade 1

overall corneal staining was a useful indicator to distinguish those with dry eye from those without dry eye.

The presentation of corneal staining in those with dry eye can vary considerably and is often monitored to review the efficacy of treatment. The corneal staining is presumed to be a sign of epithelial insult caused, at least in part, by either low tear volume or poor-quality tear fluid.[52]

To meet the new definition, symptoms of dry eye disease must co-exist with an ocular sign. The sign does not need to be corneal staining, however corneal staining does very commonly co-exist with dry eye symptoms and becomes part of the severity assessment.[56-59] However, there is little in the literature regarding the typical location of corneal staining in those with dry eye disease. Chalmers *et al*.[55] have reported that inferior corneal staining may be indicative of dry eye and Tong *et al*.[60] recently reported the inferior zone had higher staining levels at baseline in a clinical trial involving dry eye subjects. More studies are required to investigate the severity and location of staining in those with dry eye symptoms.

Another common co-existing sign in people with dry eye is conjunctival staining, which can be assessed with fluorescein, rose bengal or lissamine green. Rose bengal and lissamine green have both been shown to stain mucous strands and dead/damaged cells,[61] however rose bengal may also stain healthy cells.[62] Rose bengal stings on insertion into the eye and has been shown to be detrimental to the vitality of epithelial cells.[63] The staining appearances using rose bengal and lissamine green have been shown to be well correlated,[64] and therefore lissamine green is generally favored over rose bengal for ocular surface assessments because it is more comfortable. The conjunctival staining with either lissamine green or fluorescein is interpreted as identifying tissue that is physiologically compromised, Figure 1-18.

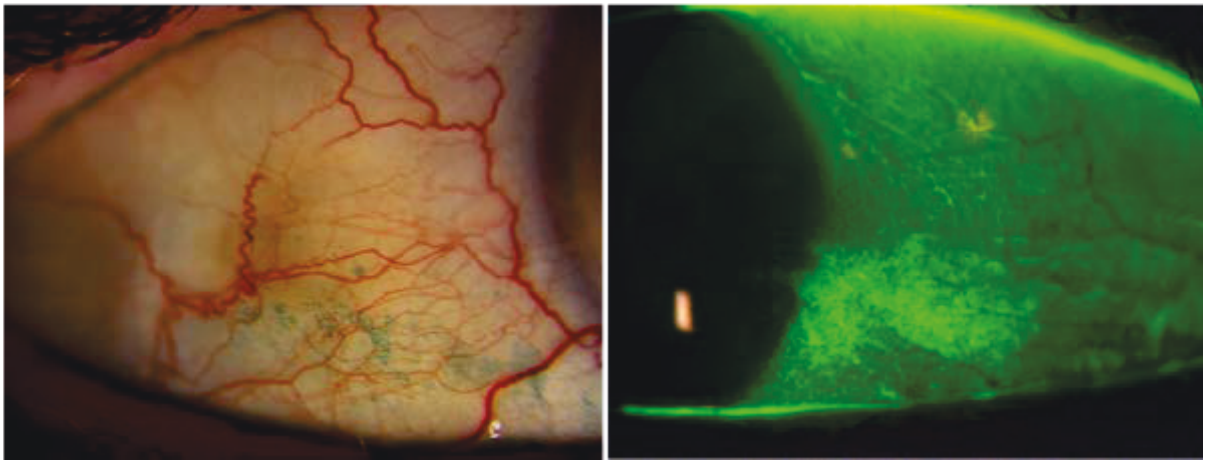

Figure 1-18: Conjunctival staining in dry eye: left image with lissamine green; right image with fluorescein. Images courtesy of CORE.

1.6 Fluorescein staining associated with contact lens wear

Corneal and conjunctival fluorescein staining is not unusual, both with and without contact lens wear.[1, 2, 65-67] The actual incidence varies across studies depending on the minimal severity reported, the scale used and the age of the subjects, however there are several characteristic staining patterns associated with contact lens wear that are frequently observed.

The conjunctiva may show a fluorescein staining pattern characteristically associated with the lens edge, which indicates tissue disruption, Figure 1-19. Also, the lens edge may create an indentation in the conjunctiva which is highlighted by the fluorescein dye because the tear layer is thicker in this indented region and therefore it fluoresces more brightly than the rest of the tear film. The indentation is described as fluorescein pooling rather than staining because it can be seen to ebb from the region of depression upon blinking. This indentation is more common in the superior region, presumably due to the upper lid creating more pressure onto the contact lens edge, Figure 1-20.

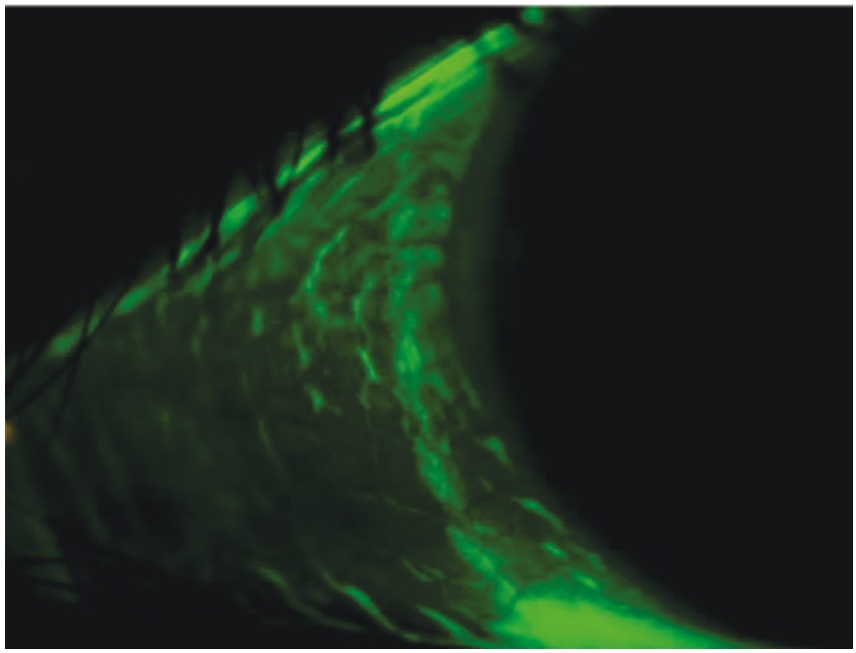

Figure 1-19: Contact lens edge associated conjunctival staining. Image courtesy of CORE.

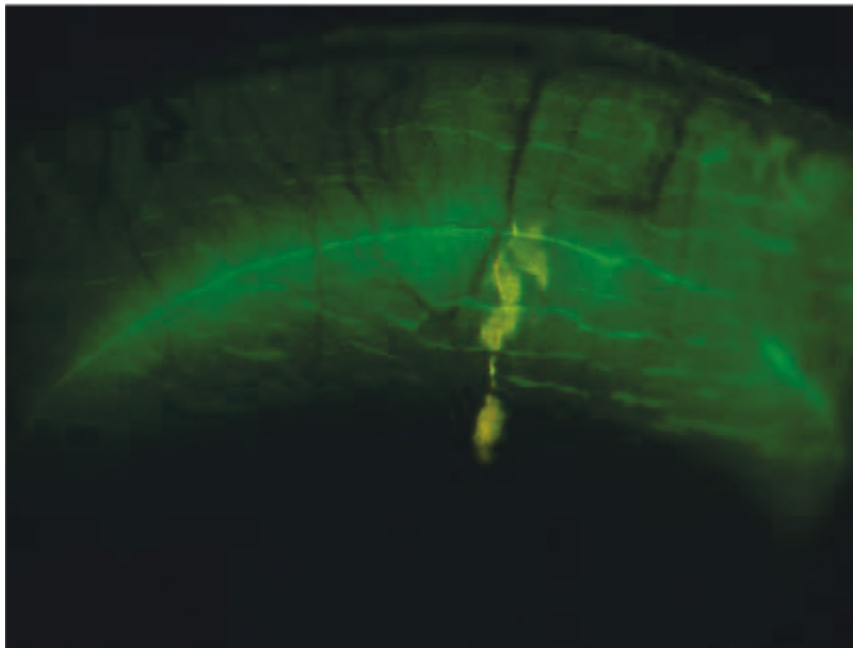

Figure 1-20: Conjunctival indentation associated with contact lens wear. Image courtesy of CORE.

For fluorescein staining of the cornea in a contact lens wearer, many of the staining patterns are indicative of specific contact lens related conditions, Figure 1-21. The staining associated with a trapped foreign body trapped under a contact lens presents as characteristic foreign body tracks or swirls as the foreign body is moved by the contact lens rotating and moving vertically with the blink action. Similar tracks may be caused by foreign bodies without contact lens wear but they tend to be smaller because the foreign bodies are swept to the inner canthus more quickly by the tear flow and lid blinking actions. A stiff lens has been known to cause superior epithelial arcuate lesions (SEALs).[26] Soft lens dehydration, sometimes associated with thin lenses, leads to the classic appearance of punctate staining in a small area in the mid-inferior corneal region, termed 'smile stain'.[68] This can also develop due to partial or incomplete blinking, which in turn causes tear film loss below the contact lens and possibly localized drying of the contact lens itself.[26] There may also be corneal staining associated with wearing a damaged contact lens. For example, a lens torn at the edge may cause conjunctival staining whereas a lens torn at the centre may cause central corneal staining. Observation of the damaged lens on eye will provide the indication of the cause for this staining.

The introduction of silicone hydrogel lenses towards the beginning of this century, brought a new characteristic fluorescein phenomena; mucin ball pooling,[69] Figure 1-21. In some wearers, the combination of lens movement with the stiffness of the silicone hydrogel material creates sheer forces that disrupt the mucin layer on the ocular surface, creating balls of mucin that are discernable on careful slit-lamp examination. These mucin balls are trapped beneath the contact lens and they become somewhat stuck to the epithelium such that they each create a small area of tissue depression.[70] When the contact lens is removed and the mucin balls are released, the instilled fluorescein fills these tissue depressions creating small pools of thicker tear film which fluoresce more brightly than the rest of the tear film, thus simulating the appearance of epithelial staining. Ladage *et al*.[71] have reported that these tissue depressions can be several cell layers thick but are not associated with cell damage or with fluorescein entering the epithelial cells. Therefore, this presentation is described as fluorescein pooling into the mucin ball depressions, not corneal staining.

There are many variations of the type and pattern of corneal staining associated with contact lens wear. No matter whether or not these staining presentations were accompanied by ocular discomfort or redness, they have always been accepted as a visual representation of damage to the corneal

surface. As such they have always triggered a call to action to either cease contact lens wear or to change the lens material or lens fit in an attempt to eradicate the corneal staining.

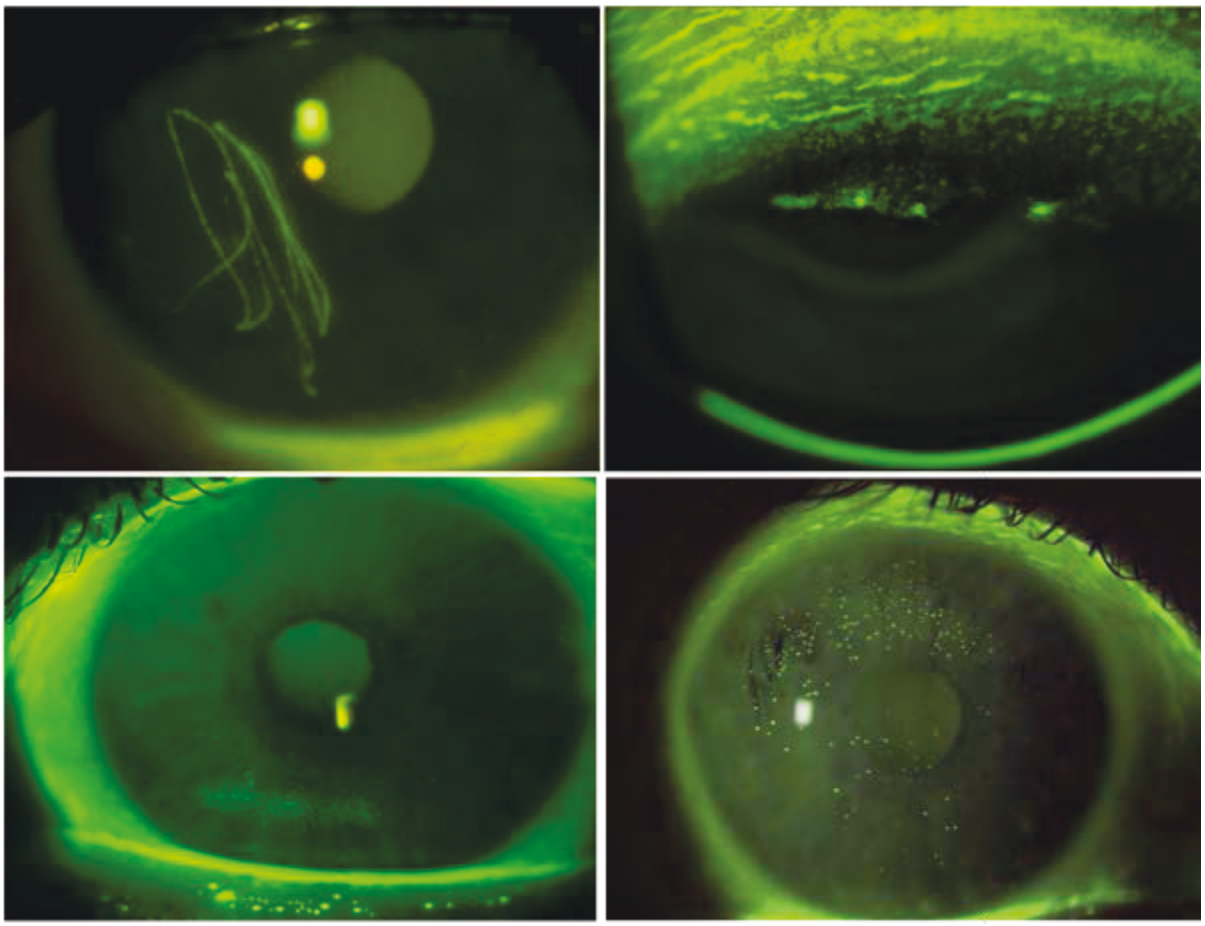

Figure 1-21: Contact lens related fluorescein patterns: top left: foreign body tracks; top right: superior arcuate epithelial staining (SEAL); bottom left: dehydration or 'smile' stain; bottom right: fluorescein pooling associated with mucin balls. Top left image courtesy of Lyndon Jones, others courtesy of CORE.

1.7 Fluorescein staining associated with contact lens care products

In the early days of soft lens care products, sensitivity to the product components was not uncommon and generally manifested with partial or widespread corneal punctate staining, together with limbal redness and ocular irritation.[72, 73] There was and still is a delicate balance between developing a

cleaning/storage product with effective disinfection and surfactant cleaning properties while minimizing epithelial cytotoxicity. Improvements in the chemical composition of care systems and the adoption of frequent replacement contact lenses in the 1990's largely eradicated this problem.[74]

The term solution induced corneal staining (SICS) was used to describe a new contact lens associated response where excessive superficial punctate corneal staining was associated with the use of, primarily, silicone hydrogel lenses and certain preserved care regimens.[75]

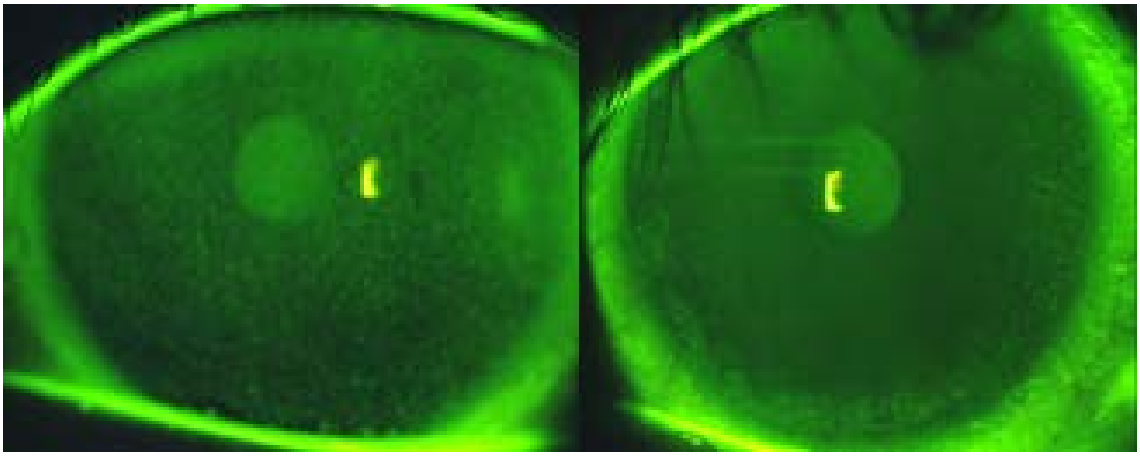

Figure 1-22: Examples of the two patterns of SICS. Left image: pan-corneal punctate staining. Right image: peripheral annulus or 'donut' pattern punctate staining. Images courtesy of CORE.

After the initial reports of this phenomenon in 2002,[76, 77] more investigations were undertaken. Garofalo *et al*.[78] conducted an investigation of four care products and three contact lenses and reported a temporal pattern to the corneal staining which was maximal between one and six hours after insertion, depending on the product combination. Following this work, several other groups investigated different combinations of lens and care products. Two of these groups created a matrix of staining responses which indicated that, although seen with several combinations, the highest levels of temporal course staining were observed when contact lenses of specific silicone hydrogel or FDA group II materials were worn for two hours following exposure to care products preserved with polyhexamethylene biguanide (PHMB).[75, 79-83] Therefore SICS appeared to be not truly product specific, but rather it was specific to certain combinations of lens materials and care systems. This has since been supported by *in vitro* work.[84] An additional unexplained factor was that products with the

same concentration of PHMB produced different levels of corneal staining when used with the same contact lens.[78, 79, 85] The SICS response has spawned many investigations into the cellular nature of fluorescein staining, which is explored further in the next section.

The corneal staining of SICS is often graded based on the area of the cornea affected[45, 49] and reporting SICS according to the area of the corneal staining seems appropriate because the staining is almost always punctate in nature and therefore it is the area of the cornea affected that is the variable factor. The extent of the punctate staining across the corneal surface describes the extent of corneal involvement. Jones *et al*.[77] described SICS as a peripheral annular pattern of punctate staining, whereas Garofalo *et al*.[78] reported a mix of annular and pan-corneal staining, Figure 1-22. Most of the subsequent studies have reported the SICS staining to be a peripheral annular (or donut-ring) pattern but some have also reported pan-corneal staining.[82, 83, 86-91] There have been no reports of whether the SICS staining pattern is repeatable within individuals. Varying the assessment time while inducing SICS repeatedly on the same individuals may answer whether the central staining has either a shorter duration or a later onset, both of which could explain the higher incidence of the reports of the annular staining pattern.

The evidence of the association of SICS with symptoms of discomfort is equivocal. The first reports by Epstein[76] and Jones *et al*.[77] described the presentation as largely asymptomatic, although Jones *et al*.[77] mentioned some reports of stinging. SICS is frequently described as asymptomatic though, when specifically reported, the symptomology varies from none,[92] through mild,[78, 79] to more clinically relevant levels.[86, 93] Situ *et al*.[94] assessed ocular surface sensitivity on eyes with SICS and found a positive correlation between the area of corneal staining and the conjunctival chemical sensitivity. Interestingly there was no correlation with corneal sensitivity. The symptomology associated with SICS is an area that would benefit from further investigation.

1.8 Cellular significance of fluorescein staining

In 1988, to explain the appearance of corneal staining, Back[95] suggested that uptake of fluorescein by the epithelial cell layers can occur under three conditions: when the tight junctions between the cells are weakened and fluorescein flows between them; when the epithelial cells are lost from the surface; when the cell membranes increase in permeability allowing the fluorescein to enter the cells.

In 1995, Wilson *et al*.[96] investigated mechanical and chemical induced staining in rabbit corneas and determined that, since some of the fluorescence did not rinse away, the fluorescence observed in fluorescein staining was intracellular and not simply pooling in the space that cells had vacated. Soon afterwards, Ward and Walker[97] demonstrated that stratified cultures of human corneal epithelial cells were impermeable to fluorescein unless damaged by chemical such as benzalkonium chloride or ethanol, highlighting the potential key role of tight junctions. Fluorophotometric techniques have been used to evaluate the permeability of the corneal epithelium to fluorescein, which provides information for the epithelial layer, rather than individual cells. In 1998, McNamara *et al*.[98] reported that overnight closure with a contact lens in place significantly increased the permeability of the epithelium, whereas overnight closure with no lens did not. Subsequent work excluded corneal hypoxia as the cause of this loss of barrier function.[99] A few years later, Miyata *et al*.[100] reported that increased levels of central epithelial fluorescein staining were correlated with increased epithelial permeability, suggesting physiological compromise.

The eruption of interest in SICS from 2002 onwards has led to renewed questioning of what corneal staining actually represents at the cellular level. Punctate staining had historically been recognized as a sign of mechanical or biochemical epithelial damage and therefore was to be avoided at all costs. The punctate staining associated with SICS was frequently at a level so alarming (see Figure 1-22) that the particular lens-product combination was replaced with another. In recent years there has been a mixture of *in vivo*, *ex vivo* and *in vitro* work to investigate the staining observed when SICS was induced by specific contact lens and care product combinations.

Bandamwar *et al*.[101] added more evidence to support the dismissal of corneal staining being caused by intra-cellular pooling of fluorescein when they reported that eye rinsing did not reduce the levels of SICS in human subjects (also see Chapter 5 and Woods *et al*.[102]). The evidence against fluorescein being able to seep between corneal epithelial cells, coupled with the findings that fluorescein cannot simply be rinsed away, leaves us with the theory that fluorescein binds to the cell membranes in some way, or it enters the cell either through diffusion or via a specific transporter mechanism.

Previous *in vitro* work by Bandamwar *et al*.[103] reported that live healthy cells absorbed fluorescein to a low level that did not give rise to fluorescence, a theory more recently supported by others.[104, 105] The cellular uptake of fluorescein was shown to be an active process, as only metabolically active cells stained with fluorescein while necrotic (dead) cells were unstained.[104, 106] Bakkar *et al*.[104] furthered the theory that active transport was involved in fluorescein staining by demonstrating that

fluorescein uptake was reduced when cells were exposed to low temperatures (4°C). Furthermore, Bandamwar *et al*.[103] reported that confocal examination of rabbit corneas with induced SICS revealed that the cells taking up fluorescein were in early stages of apoptosis because they also stained with Annexin V. This apoptotic state of fluorescein stained cells was later supported by Gorbet *et al*.[107] who used the presence of activated caspase to confirm apoptosis.

Recent work has demonstrated that the 'dots' of fluorescence seen in punctate corneal staining actually correspond to individual epithelial cells[106, 108] and that the fluorescein is found on the cell membrane as well as within the cell, sometimes even within the nucleus[104, 107, 109] , Figure 1-23.

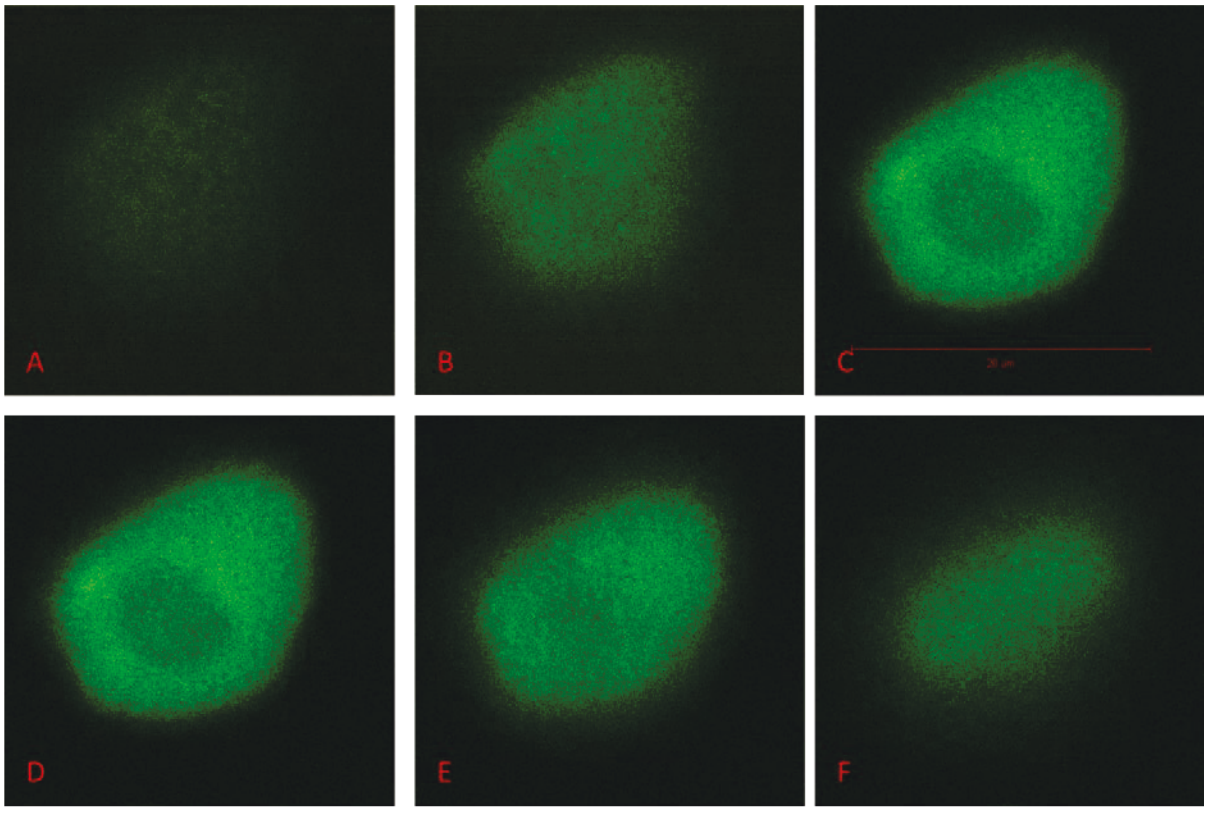

Figure 1-23: Diffuse fluorescein is observed throughout the cytoplasm and nucleus of an epithelial cell collected from an eye with SICS. Confocal microscope images at depths in 4μm steps from 4μm (A) to 24μm (F). Image reproduced from Gorbet M, Peterson R, McCanna D, Woods C, Jones L, Fonn D. Human corneal epithelial cell shedding and fluorescein staining in response to silicone hydrogel lenses and contact lens disinfecting solutions. Curr Eye Res. 2014;39:245-56.[7]

This body of work together with the more recent work by Khan *et al.*[105] have confirmed the involvement of some specific cellular transport mechanisms and that cellular damage (apoptosis) is associated with SICS. These findings are in contradiction to a recent theory about the passive nature of SICS. The latter theory is referred to as "preservative associated transient hyperfluorescence", or PATH,[110] and it was developed from in-vitro work using a liposome-based model of the corneal epithelium. The PATH theory proposes that corneal staining in SICS is not evidence of epithelial damage, suggesting instead that PHMB molecules released from the contact lens bind to phospholipids in the bi-layer cellular membrane of the corneal epithelial cells, and fluorescein then binds to the phospholipid-PHMB complex and fluoresces.[111, 112] Thus the PATH theory concludes that the fluorescein-PHMB-epithelial cell binding is inert and does not lead to any cellular compromise. Preceeding work by Muya *et al.*[113] had identified an affinity for PHMB to bind to the mucins found on the epithelial surface. However, despite some support for the PHMB binding process, PATH does not explain why fluorescein is also seen within the epithelial cells[104, 107, 109] and appears to be associated with apoptosis.[103, 107]

A recent report by Khan *et al.*[105] questions the previously presumed link between SICS and PHMB and instead presents evidence of a relationship between SICS and the surfactant Tectronic 1107, which co-exists with PHMB in SICS inducing care products. This *in vitro* work demonstrated increased fluorescence of epithelial cells when they were exposed to Tectronic 1107. These results implicate a dynamin dependant pathway (an active transport mechanism) as responsible for the increased fluorescence, but the authors did not observe apoptosis associated with fluorescein uptake in their cells, thus casting new doubt on the fact that fluorescein staining was associated with cellular damage. Variations in the source of corneal epithelial cells and in experimental protocols are just two possible explanations why contradictory results are being reported

Apart from fluorescein staining, two other epithelial cellular changes have been observed in corneas that exhibit SICS: epithelial 'white light' staining is evident,[101, 108] and hyper-reflective epithelial cells have been observed using confocal microscopy.[106, 114-116] Both of these observations are reported prior to fluorescein instillation and therefore cannot be explained by a fluorescein binding action. Their observation in corneas that are subsequently positive for SICS suggests they are a cellular response to the lens/care product combination.

The ‘white light’ staining can be observed on careful observation with a slit-lamp biomicroscope, Figure 1-24. Pale grey punctate disturbances of the epithelium can be seen in the same region that is highlighted with any subsequent fluorescein instillation.[101, 108].

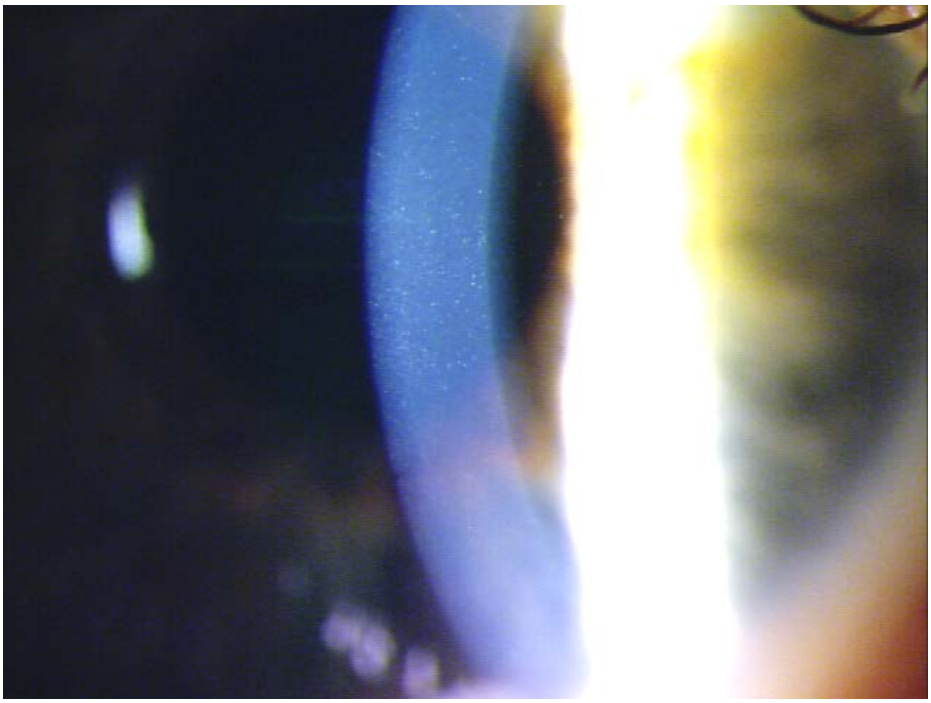

Figure 1-24: Appearance of ‘white light’ corneal staining using a slit-lamp. Image courtesy of CORE.

The term hyper-reflective epithelial cells refers to individual highly reflective superficial epithelial cells that can be viewed using *in vivo* confocal microscopy, Figure 1-25. Their high level of reflectivity has been suggested to indicate that they are apoptotic,[103] and they have been reported to be visible in association with SICS.[106, 114-116] The anesthetic required for confocal viewing has been highlighted as a confounding factor, however Situ *et al*.[116] reported on the association of hyper-reflective cells with SICS when imaging was conducted over the contact lens, eliminating the use of an anesthetic.

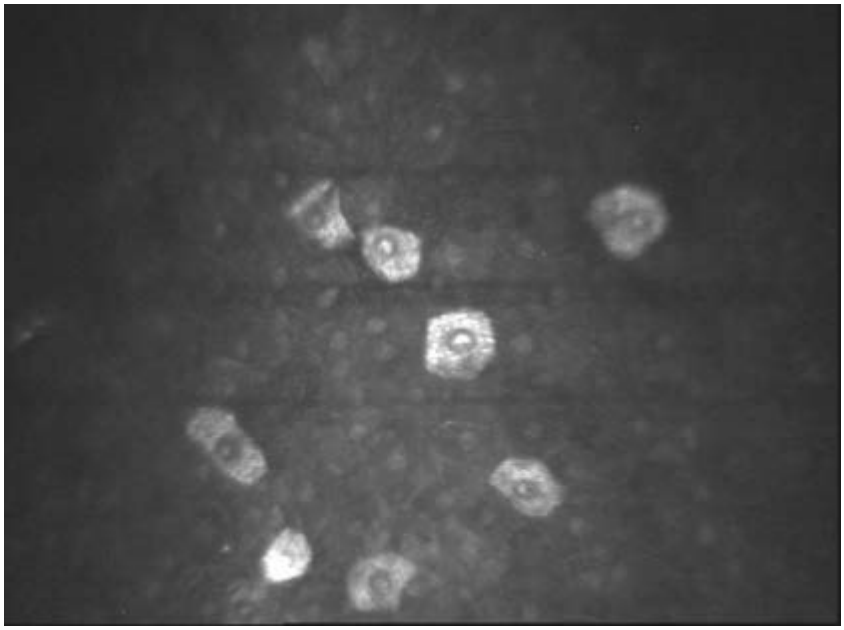

Figure 1-25: Hyper-reflective surface epithelial cells of the cornea. Confocal microscope *in-vivo* image, courtesy of CORE.

These clinical and laboratory findings have led to widespread recognition that there is a lot still to be discovered about fluorescein interaction with the epithelium, despite it being interpreted and relied upon for over a century.[35, 62] There is general agreement that corneal fluorescein staining is not desirable, yet despite a significant body of work investigating it, this belief is based largely on "*assumption and intuition*".[35] It is fair to say that how fluorescein interacts with the cornea at the molecular level remains unclear[35, 62, 117] and further investigation is warranted in order to fully understand the clinical implications of epithelial fluorescein staining.

1.9 Aims of the thesis

The reviews of the literature described above demonstrate that there are three very specific areas concerning corneal staining with fluorescein that warrant additional study. The specific aims of this thesis are:

a) To determine whether the corneal staining of subjects with symptomatic dry eye presents in a specific distribution pattern;
b) To investigate the type, severity and pattern of staining that occurs in SICS;
c) To assess the impact of SICS on individual epithelial cells using in vivo confocal microscropy.

This thesis is organized into seven chapters:

Chapter 2 describes a grading scale developed for grading corneal staining; the CORE corneal staining scale. This chapter describes this 3-factor grading scale, which is the method of staining grading used in the studies featured in Chapters 3, 5 and 6.

Chapter 3 examines the CORE corneal staining scale for agreement across observers with various experience.

Chapter 4 presents a meta-analysis of the corneal staining data across several clinical studies involving 368 subjects with symptomatic dry eye. The data were analysed to determine if there was a typical staining pattern in subjects who were symptomatic of dry eye. This information will be valuable in setting meaningful treatment efficacy targets for the evaluation of new dry eye treatments and therapies.

Chapter 5 reports a three phase experiment designed to evaluate some of the elements of SICS with respect to the PATH theory. The first two phases investigated whether the PHMB and other lens care components could be either rinsed from the lens before it was inserted to the eye, or rinsed from the eye after lens removal, thereby reducing the level of SICS staining. The third phase investigated cellular changes in the presence of SICS by employing *in vivo* confocal microscopy to investigate the incidence of hyper-reflective epithelial cells.

Chapter 6 presents two analyses of the data from several studies that induced SICS using the same products and methodology. Firstly, the frequency of central zone involvement is evaluated to explain the frequency of peripheral annular pattern staining versus the pan-corneal pattern staining. Secondly,

data from participants enrolled in repeated SICS studies were evaluated for the repeatability of the corneal staining pattern.

Chapter 7 discusses the previous chapters, concludes the findings. Suggestions for future work are presented.

Chapter 2
The CORE Corneal Staining Scale

THIS CHAPTER IS PUBLISHED AS FOLLOWS:

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2.1 Abstract

The assessment of corneal staining is a commonly conducted procedure in both clinical practice and as part of various research studies. Different grading scales are employed by many clinicians and researchers to undertake this procedure for corneal staining comparisons between eyes, products and over time. This paper describes the development and use of a grading scale for corneal staining undertaken at an academic research site. The scale employs assessment of three factors across five corneal zones; type, area and depth. Staining type and area are graded on a 0-100 scale and depth is graded on a 0-4 scale. These factors can be combined to create a 3- or 2-factor staining grade, or the factors may be reported individually. An additional benefit of this scale is that the staining scores may be reported by zone as 'zone staining scores', or the scores of zones may be combined to provide an overall corneal 'global staining score'.

2.2 Introduction

Over the past few years there has been dispute regarding the conclusions that can be drawn from the presence of sodium fluorescein corneal staining.[1-3] Despite questions about the clinical significance of corneal staining, particularly that associated with contact lens solutions,[1, 2] this assessment remains an essential element of ocular examination and thus the ability to record the level of corneal staining is important in clinical practice and is vital in contact lens and dry eye research.

Unfortunately, methods of assessing corneal staining are varied in their approach and there is a need for this assessment to be standardised.[4] While there has been growing interest in the development of objective methods to quantify staining and other anterior eye assessments,[5-8] these have yet to become mainstream and to-date none have been commercialised.

The challenge is to develop a standardised method that provides high sensitivity to detect change. Limitations of current scales prompted discussion within the Centre for Ocular Research & Education (CORE), formerly the Centre for Contact Lens Research, around the creation of a new corneal staining grading scale that would provide advantages over those available. This scale has evolved over several years, being first described in any detail in a paper investigating solution-induced staining with silicone hydrogel lenses in 2002[9] and further described by Woods and co-workers in 2006.[10] This paper describes further evolution and uses of the CORE staining scale.

2.3 Scale development

When describing and quantifying corneal staining, ideally three elements need to be considered: location, area and depth.

There are many different corneal staining grading scales currently in use. Examples of those that provide an integer grade for the entire corneal surface include Oxford,[11] and Sjogren's Syndrome International Registry scale (SICCA OSS).[12] These scales offer varying ranges for this global corneal grade; 0-4, 0-5 and 0-6 integer ranges, respectively. Developed to provide more sensitivity, the Efron corneal staining scale[13] is a 0-4 global corneal scale which can be used in steps as small as 0.1. Other staining scales are based on grading the five corneal zones separately (Figure 2-1).[14] Examples of scales which employ this zonal grading include the US Food and Drug Administration (FDA), the National Eye Institute (NEI) and the Brien Holden Vision Institute (BHVI) scales. In 1994 an FDA guidance document to the contact lens industry suggested that reporting the location of corneal staining would be beneficial, to allow distinction of central staining from peripheral location and they suggested a 0-4 integer scale followed by a letter code for the zone affected.[15] The NEI report of 1995[4] also suggested a grading by zone, but suggested a 0-3 integer scale.

The BHVI Grading Scales (formerly the CCLRU Grading Scales) were first described in 1993[16] and included a multi-factorial corneal staining grading scale, which offered an increased number of

steps compared to previous scales with the intent to provide increased sensitivity.[17] This scale involved assessment of staining in each of the five corneal zones but instead of just one grade, it included three separate staining factors; type, extent and depth. Each criterion is scored on a 0-4 scale, with steps as small as 0.1.[18] The BHVI three-factor scale provided increased descriptive ability compared to a single number scale. However, despite being more descriptive, and providing a greater ability to detect change compared to other reduced step scales, it's sensitivity was still somewhat limited by the 41 steps of the 0-4 scale in 0.1 steps and by the separate reporting of the three factors. These limitations prompted the creation of the CORE corneal staining scale.

Scales with a small number of steps, such as the Oxford (0-3 integer) and NEI scale (0-15 integer), typically have good repeatability (Intraclass correlation coefficient of 0.97 and 0.98),[19] however they lack sensitivity.[20] Creating a scale with more steps can create the opposite problem i.e. improve sensitivity but tend to reduce repeatability.[19]

The purpose behind developing the CORE corneal staining scale was to address several needs:

- increased sensitivity compared to 0-4 scales.
- enable the location of the staining to be recorded.
- facilitate grading of individual criteria of the staining.
- to generate continuous data to facilitate parametric data analysis.

It was decided to utilize the division of the cornea into five zones, each to be assessed separately: the central (C), superior (S), nasal (N), inferior (I), and) temporal (T) zones (Figure 1). This zone division had already been described[14] and has been supported as being beneficial by a subsequent global versus zonal grading comparison.[21]

Despite zonal division of the cornea having been described previously, the diameter of the central zone relative to the cornea has not been specified. Some have described the zones as being of equal or similar size, but have stopped short of being more specific than that.[16, 17] In order for the CORE scale to be applicable to all size eyes and in all clinical situations, the central zone was specified to have a diameter that is one half of the diameter of the cornea. This proportion allows the observer to readily visualise the central zone as distinct from the peripheral zones, because from the centre of the cornea, the central zone extends half way to the limbus in all directions. This still means that the five zones have a similar area, though the central zone is slightly larger than the peripheral zones. For example for a corneal diameter of 11.51mm, which is the average of mean Chinese and Caucasian corneal

diameters reported by Hickson-Curran et al,[22] the area of the central corneal zone is 22.53mm^2, and the area of each peripheral corneal zone is 20.77mm^2 (using $\prod = 3.142$). For larger corneas the difference in area will increase.

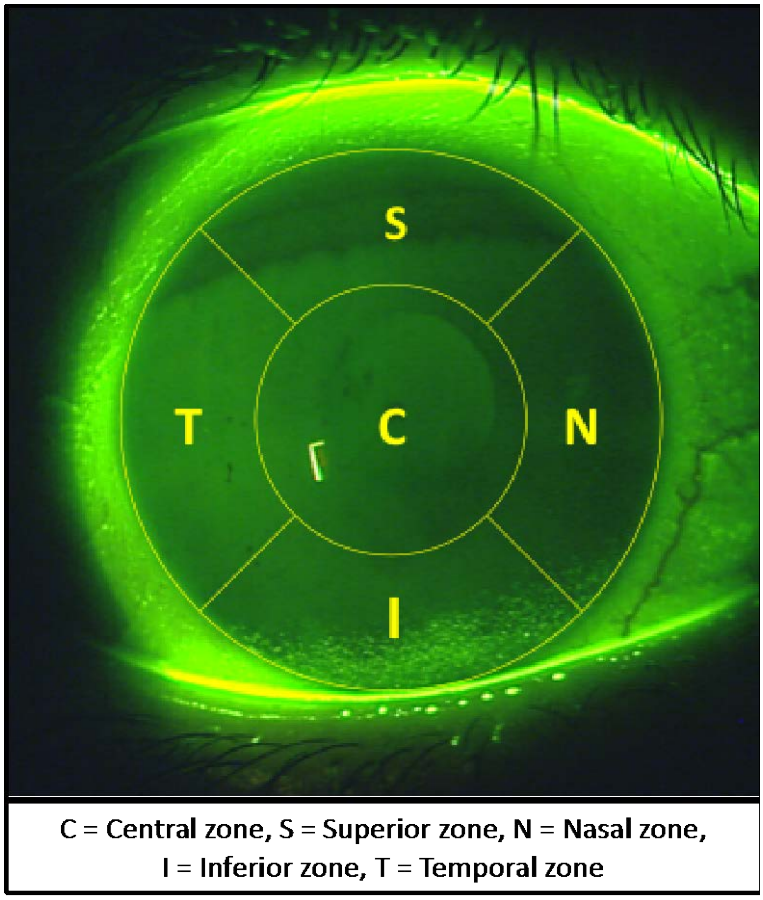


Figure 2-1: Schematic illustration of the five corneal zones.

In order to describe the staining as fully as possible it was decided to follow the same multi-factor approach as the BHVI grading scale and describe the same three distinct factors of the staining appearance, separately across each of the five zones:

1. the ***type*** of staining,
2. the ***extent*** (area) that the staining is spread across,
3. the ***depth*** of the staining.

Consideration was given to expanding the scale to improve sensitivity and create continuous data points that, if normally distributed, would facilitate parametric analysis.

The ***type*** of staining was allocated a continuous integer scale, from 0 to 100 with anchors as described in Table 2-1 and illustrated in Figure 2-2.

Table 2-1: CORE staining grade for the type of fluorescein staining, 0-100 integer scale.

Grade of TYPE of fluorescein staining	Description
0	No staining
25	Micro-punctate staining
50	Macro-punctate staining
75	Coalescent staining
100	Patch staining

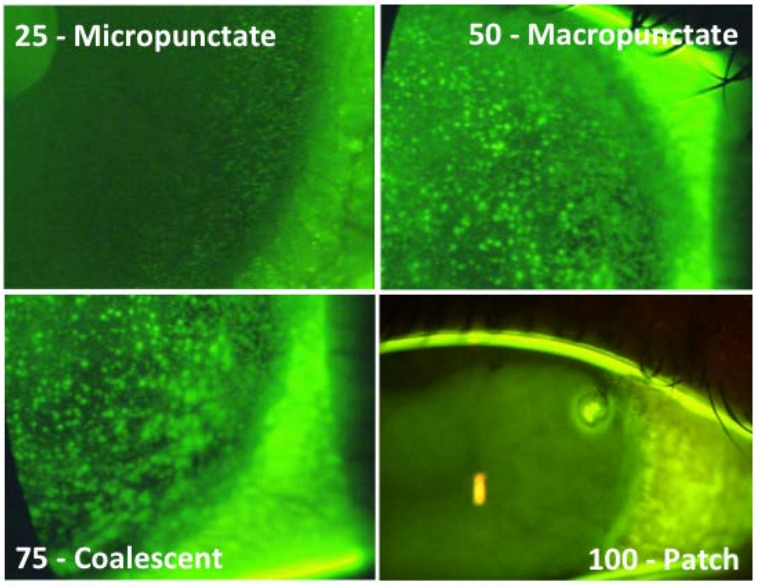


Figure 2-2: Image references for staining types.

The ***extent*** of staining was also allocated a continuous integer scale, from 0 to 100, to represent the percentage area of the individual zone that contains corneal staining. One important feature of the ***extent*** grade is that it does not represent the area of all the punctate dots pulled together, but rather it represents the spread of the staining across the zone (see Figure 2-3). For example, if punctate stain was evident across the entire central zone then the extent within that zone would be represented by 100, despite the staining dots being small and spread apart from each other.

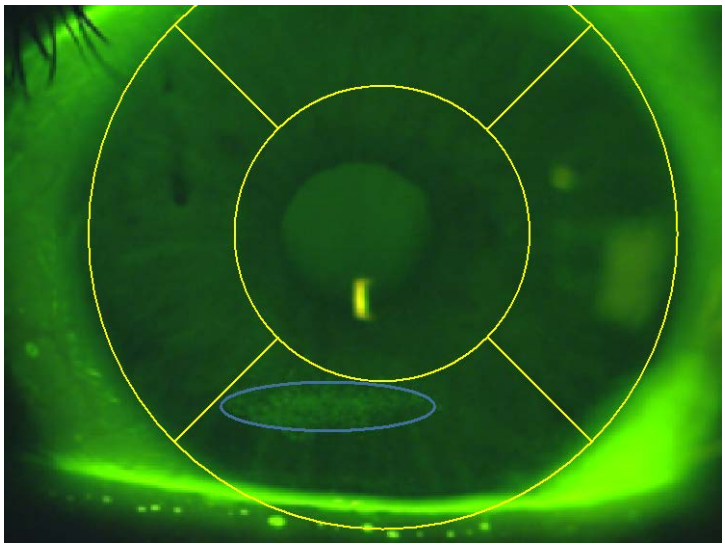


Figure 2-3: The blue outline illustrates the extent to be allocated to punctate stain, Extent=20.

The ***depth*** of corneal staining was already a part of the BHVI grading scale in the form of a 0-4 integer scale with distinct descriptors. Given the limited options available to describe the depth of staining in terms of the penetration of fluorescein into the cornea, this scale was determined to be sufficient for grading the depth of staining and was adopted into the CORE staining scale (Table 2-2).

Table 2-2: CORE staining grade for the depth of corneal staining, 0-4 integer scale.

Grade of DEPTH of fluorescein staining	Description
0	No staining
1	Superficial epithelial; no stromal glow
2	Deep epithelial; delayed stromal glow
3	Immediate localised stromal glow
4	Immediate diffuse stromal glow

2.4 Application of CORE corneal staining scale

Because the central zone has a diameter that is approximately half of the diameter of the cornea for ease of assessment, then the central zone has a slightly larger area than each of the other four zones.

2.4.1 Clinical records

When each zone is graded fully to describe the presence of staining then three values are generated for each zone, as shown in the recording form example in Figure 2-4 i.e. for type (1-100), extent (1-100) and depth (1-4). These values are all that is required on a clinical record to describe the staining appearance in each corneal zone and they provide a simple three-factor description, which facilitates monitoring and comparison for change at subsequent clinic visits across all three factors of type, extent and depth.

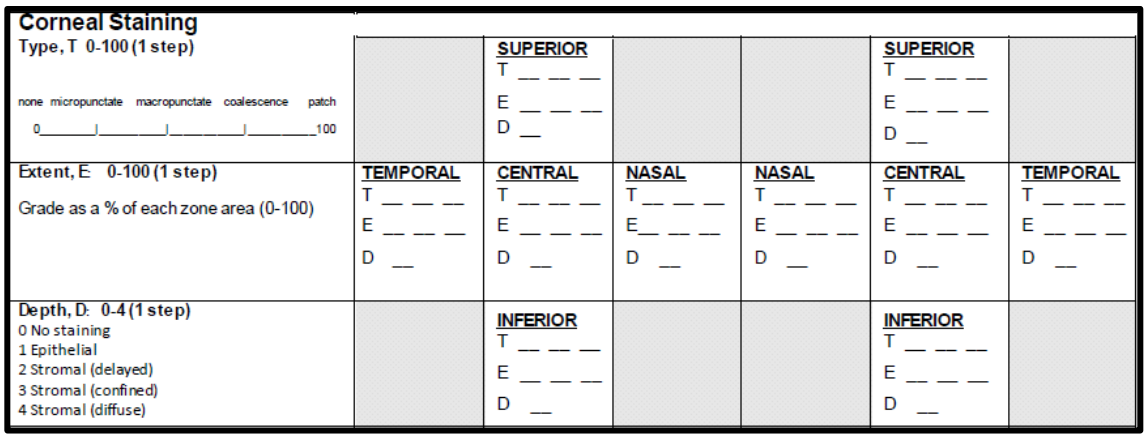


Figure 2-4: An example of a clinical recording table for CORE corneal staining scale.

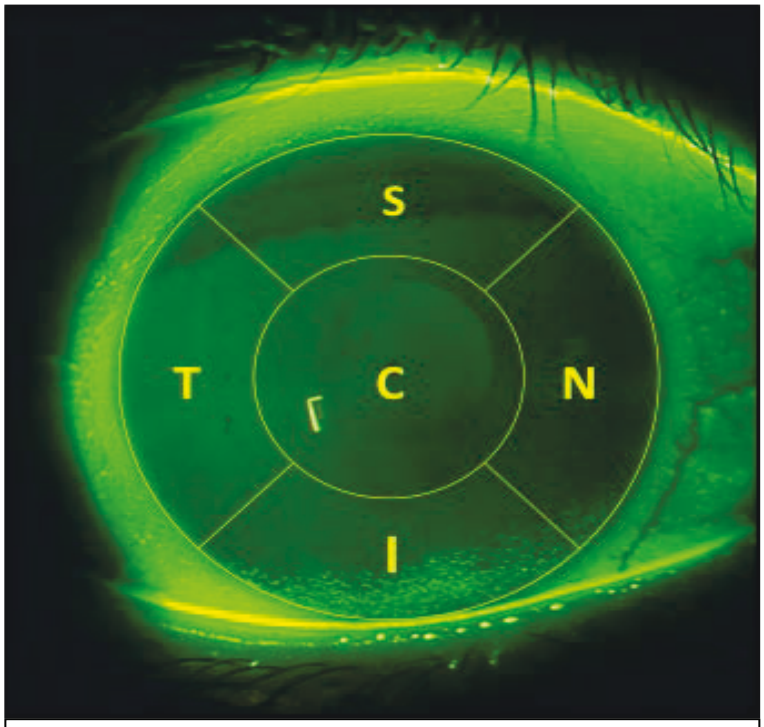
2.4.2 Reporting the staining

The staining can be reported and analysed for the individual zones or for the entire cornea as a whole. The scores for the three factors were combined by three-way-multiplication to create an even wider spread of data to increase sensitivity. The individual zone score is referred to as the **"zone staining score", ZSS,** and the scores are abbreviated as CZSS, SZSS, NZSS, IZSS and TZSS for the central, temporal, nasal, superior and inferior zones respectively. The mean of all five zone stain scores provides the **'global staining score', GSS,** which represents the cornea as a whole.

The three values that make up the staining grade can be manipulated in several ways to report the staining, depending on the distribution of the data and the key factor of interest among the three factors. The three common options are listed here.

3-FACTOR REPORTING OF CORE CORNEAL STAINING SCALE:

The product of the type, extent and depth grades provides the 3-factor grade for each corneal zone, and in this case the ZSS and the GSS are scored out of a maximum of 40,000 (0-100 x 0-100 x 0-4), see Figure 5.



Zone stain score = type x extent x depth

CZSS	= 0 x 0 x 0 = 0
SZSS	= 0 x 0 x 0 = 0
NZSS	= 60 x 8 x 1 = 480
IZSS	= 65 x 45 x 1 = 2925
TZSS	= 20 x 2 x 1 = 40

Figure 2-5: An example of staining and the representative ZSS's and GSS.

2-FACTOR REPORTING OF CORE CORNEAL STAINING SCALE:

Most corneal staining seen in clinical contact lens research, other than in cases of an adverse event, has a depth of 1 (superficial epithelial), resulting in this extended grading scale effectively being truncated back to a 2-factor grade, which provides a 0-10,000 scale range. In this case the ZSS and GSS are effectively the product of just two grading values i.e. type and extent, because the depth grade is always 'grade 1'. In this method the total staining grade could reach a maximum of 10,000.[9, 23, 24]

In studies where variability in the staining depth is anticipated then adding the depth as a third factor to reach a full 3-factor staining score (type x extent x depth), as described above, will serve to capture the staining more fully and to spread the staining data even wider to aide differentiation between eyes/treatments. In practice, if all staining is superficial (i.e. depth grade is 1) then the 2-factor grade yields the same value as the 3-factor grade.

1-FACTOR REPORTING OF CORE CORNEAL STAINING SCALE:

There are occasions when the CORE scale has been used to simply report the extent of corneal staining over the corneal surface, either by zone or over the cornea as a whole.[25] In specific clinical research projects where the depth of staining is always grade 1 and the type of staining is consistent or of limited interest, it is the extent of the staining that is the key outcome variable. For example, if the corneal staining is micro-punctate in all eyes, and is superficial epithelial in all eyes, then the variability of the staining score is captured by the extent grade alone. Reporting extent alone has the advantage of being easily interpreted by the reader because it can be readily 'visualised' as the area of the zone, or cornea, which exhibits staining.

2.5 Discussion

This grading scale has been used extensively at CORE for over a decade, largely because it is flexible and lends itself to multiple methods of representing the staining numerically and statistically. The expanded scale of 0-100 for the type and extent, and also the manipulation to create the zone and global staining scores, all provide a continuous scale for grading the corneal staining. The continuous nature of the data opens the possibility for conducting parametric statistical analysis, which is often favoured as being more robust. It also affords the potential for increased sensitivity, which is of particular benefit when attempting to discriminate responses in clinical trials.

There are some disadvantages to increasing the number of steps in a scale. For example, individuals may prefer to use certain numbers more often than others, thereby reducing the number of steps being utilised. It was reported by Fieguth and Simpson that an integer step scale of 0-100 gave rise to a preferential use of numbers that were multiples of five.[26] Their investigation involved a bulbar redness scale, but the preferential use of 'friendly' values could also apply to this corneal

staining scale. It would perhaps be valuable to conduct a review of historical staining data to investigate if this is the case. Another disadvantage of creating a scale with more steps is that repeatability and inter-observer concordance is often sacrificed. For this reason the use of this scale among multiple observers necessitates pictorial references, particularly for the staining type, and ongoing concordance assessment and training.[18] Variability may exist between different graders (inter-variability), as well as between different time-points for the same grader (intra-variability). Using a grading scale to assign a grade to an eye is an inherently subjective task. The key to any subjective grading scale being used successfully is to *minimise* inter- and intra-variability, because it is impossible to eliminate it. This can be achieved in both a practice and a research setting by asking graders to grade a randomised series of images on two separate occasions. The viewing conditions should be controlled between sessions as much as possible, such as using the same room and same computer screen. The grading results should be compared and retraining conducted where necessary. Variability is a criticism of all grading scales which rely on subjective assessment by an observer.[18, 27] A grading scale which is truly objective, by means of image analysis, can certainly reduce the variability, but it becomes more costly in terms of supporting hardware and software, which can be prohibitive in a clinical versus research setting.[5-8] Furthermore, there is often increased time required to apply the software and review the area of interest identified by the algorithm, before a grading score can be confirmed.[5-8]

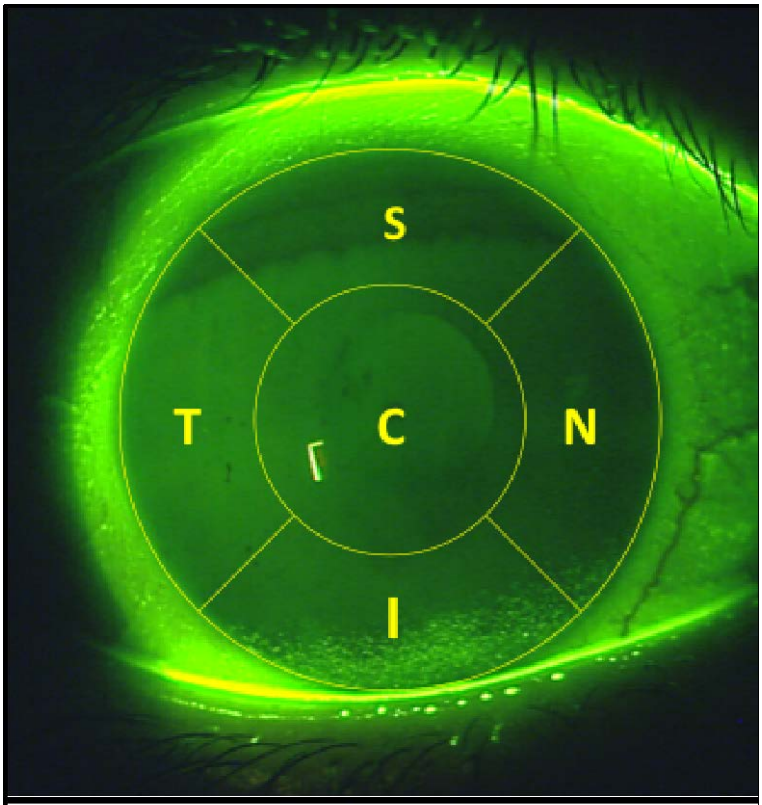
The CORE corneal staining scale provides the ability to record the type, extent and depth of staining by zone or globally across the entire cornea. Using zone scores it facilitates the reporting of the location of corneal staining. It is often important to understand where on the cornea the staining is observed, and this information is unavailable in grading systems that provide only a single grade for the whole cornea. For example mid-inferior dehydration type staining and superior epithelial arcuate lesions both have specific and different implications for a soft contact lens design, and require different management strategies.[28]

The CORE corneal staining scale has proven to be useful when the location and/or the quantification of corneal staining are of key interest, and when more robust, parametric analysis has been desirable. Additionally, the scale provides the level of sensitivity often necessary in clinical research, while being simple enough to be adopted in a clinical practice setting. The authors believe the CORE scale has potential to become a broadly used grading system for recording corneal staining.

Chapter 3
Agreement Experiment: The CORE Corneal Staining Scale

3.1 Introduction

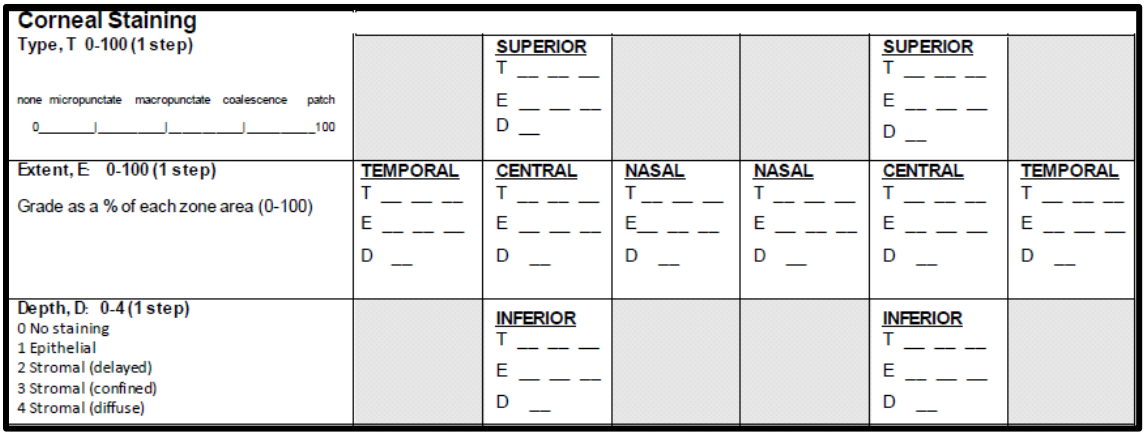
Fluorescein corneal staining is presumed to indicate sub-optimal physiology or damage.[1] A grading scale to record the severity or extent of this staining is valuable to monitor recovery from injury, or to measure any negative impact of a medical device like a contact lens. The CORE corneal staining grading scale is described in detail in Chapter 2. It is a 3-factor scale which grades the ***type***, ***extent*** and ***depth*** of corneal fluorescein staining,[2] in each of the five corneal zones; temporal superior, nasal, inferior and central,[3,4] Figure 3-1.



C = Central zone, S = Superior zone, N = Nasal zone,
I = Inferior zone, T = Temporal zone

Figure 3-1: Schematic illustration of of the five corneal zones.

The type and extent of the staining are graded on a 0-100 integer scale. The type of staining has descriptors at the 0 (none), 25 (micropuncate), 50 (macropunctate), 75 (coalescence) and 100 (patch) intervals of the scale, Figure 3-2.



Corneal Staining						
Type, T 0-100 (1 step) none micropunctate macropunctate coalescence patch 0_____|_____|_____|_____|_____100		**SUPERIOR** T __ __ __ E __ __ __ D __			**SUPERIOR** T __ __ __ E __ __ __ D __	
Extent, E 0-100 (1 step) Grade as a % of each zone area (0-100)	**TEMPORAL** T __ __ __ E __ __ __ D __	**CENTRAL** T __ __ __ E __ __ __ D __	**NASAL** T __ __ __ E __ __ __ D __	**NASAL** T __ __ __ E __ __ __ D __	**CENTRAL** T __ __ __ E __ __ __ D __	**TEMPORAL** T __ __ __ E __ __ __ D __
Depth, D 0-4 (1 step) 0 No staining 1 Epithelial 2 Stromal (delayed) 3 Stromal (confined) 4 Stromal (diffuse)		**INFERIOR** T __ __ __ E __ __ __ D __			**INFERIOR** T __ __ __ E __ __ __ D __	

Figure 3-2: Example of staining record sheet, which includes the descriptions associated with the grade of the type of staining. Image courtesy of CORE.

The extent of the staining is a grade of the area of the corneal zone affected by the staining. Punctate (dot appearance) staining is not graded as if it were confluent packing together of the punctate dots. Instead the border of the area of punctate staining is assumed to outline the area of cornea affected, Figure 3-3.

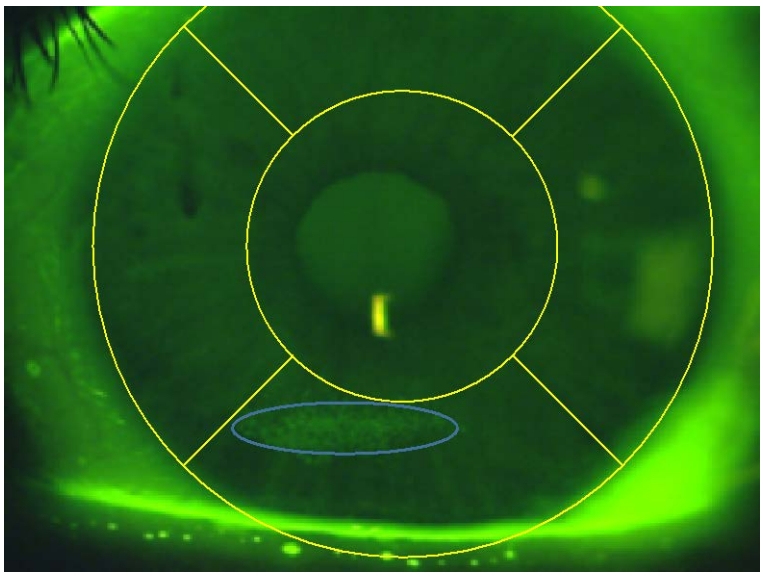


Figure 3-3: Illustration of the border defining the extent of staining. Image courtesy of CORE.

The depth of the staining is graded on a 0-4 integer scale and is assessed according to the observation of fluorescein into the stromal corneal layer and the speed of this stromal spreading, if any, which is often termed 'stromal glow', Figure 3-4. Fluorescein that remains within the epithelial corneal layer is graded as depth of 1. Where the corneal insult is deeper than the epithelium, the fluorescein seeps gradually into the stroma and is visible as a diffuse glow around discrete region of epithelial staining. The faster this glow develops, the deeper the corneal insult. Therefore, the depth of corneal staining cannot be assessed from a single photographic image because it is not possible to make a temporal judgement.

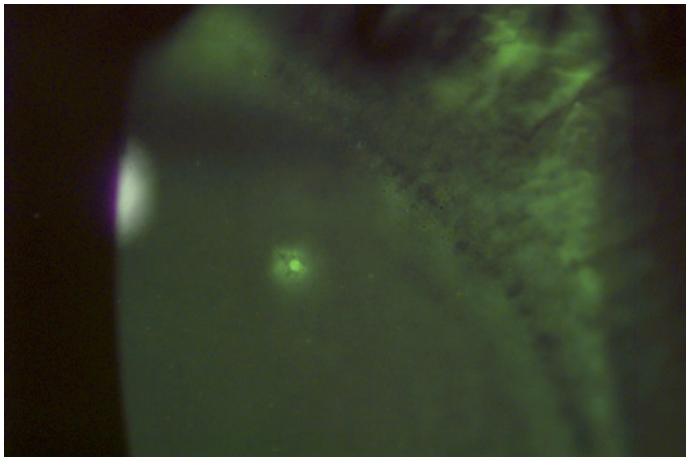


Figure 3-4: Illustration of the stromal glow around the discrete punctate stain, indicative of a corneal insult that is deeper than the superficial epithelium. Image courtesy of CORE.

Understanding how a grading scale behaves is valuable. In clinical healthcare and research it is recommended to conduct regular training to confirm that the measurement skill is maintained and optimised.[5,6] This training may involve the practical methodology involved in making an assessment, as well as evaluating and discussing the grading results to determine accuracy and repeatability.[2] In an ideal world, clinical assessments would be accurate (match the correct answer) and repeated measurements would be precise (repeated measurements would demonstrate good agreement and only differ when there is an actual difference in presentation of the staining).

There is an element of subjectivity involved when using grading scales as they involve matching a clinical presentation to a numeric, pictorial or descriptive scale.[6,7] A judgement is required in order to decide which scale value best matches the clinical presentation. This element of subjectivity has the potential to reduce both accuracy and precision because this judgement made by the observer unavoidably incurs 'noise', rather than there being an absolute value to measure.[6] The judgement of an observer may be biased due to clinical experience and the level of knowledge of the particular scale being used. For example, a red eye may be graded as 'severely' red by a clinician who rarely sees any red eyes in their practice, yet may be graded as 'moderately' red by a clinician who only treats red eyes with infections. A reference pictorial scale of eye redness would allow these two clinicians to scale their judgements of the redness similarly and more accurately (ie. they grade correctly, according to the scale).

It is logical that it is easier for clinicians to record the same grade for the same condition if there are fewer grading options. From the earlier example, if the red eye grading options were simply 'yes' or 'no', the clinicians would have both recorded 'yes', and they would have been both precise and accurate. However, a binary scale cannot indicate the degree or severity of the condition, such as redness, and this missing factor is often necessary for determining urgency of treatment or monitoring response to treatment, particularly when a different clinician conducts the post-treatment follow-up. Determining the number of steps in a grading scale can be a complex decision. A scale with too few steps allows the clinicians to be precise, also called repeatable, but does not provide many options to describe the severity and therefore these scales are not very sensitive to changes in severity. Increasing the number of steps will increase the options for descriptions, but may also reduce the precision, or repeatability both between different clinicians as well as for the same clinician on different days.

The repeatability of a scale can be measured by comparing how an individual observer grades the same condition on two different occasions. This comparison is often referred to as the intra-observer agreement. By increasing the number of observers, it is possible to assess whether the scale performs in a reliable manner between different observers, often called the inter-observer agreement. In both cases, a low level of agreement indicates the scale is not performing optimally and that either the scale requires modification or the observers require training on how to better use the scale, or perhaps both. Agreement is often described by correlation indices, and these range from 0.0, which indicates there is no agreement at all, to 1.0, which indicates perfect agreement. While the criticality of the measurements being assessed will influence the acceptable value of this index, the commonly accepted interpretation of these correlation indices are described by Lin[8] as:

> 0.99	almost perfect
> 0.95 – 0.99	substantial
0.90 – 0.95	moderate
< 0.90	poor

The subjective nature of grading scales may present an argument for a less strict interpretation of the correlation index values. However, it should be remembered that a value of 0.75 indicates that any inferences or conclusions drawn are only supported by 75% of the data, therefore it is unwise to make the interpretation overly loose when deciding what value represents substantial and moderate agreement.

In research specifically, if the variable is to be measured by different observers then inter-observer agreement should be understood and minimised. Experiments designed to measure differences are obviously more sensitive when the methods of measuring are more precise. If inter-observer agreement is poor and intra-observer agreement is better, then, if possible, it would be preferable to design the experiment such that the same observer grades the response in the same subject over time. Analyses of agreement are important and the results provide valuable information to support considerations of study design as well as the interpretation of results.

A repeatability experiment was conducted using the CORE corneal staining grading scale. The key objectives were to evaluate the inter- and intra-observer agreement of the grades for type and extent of the corneal staining.

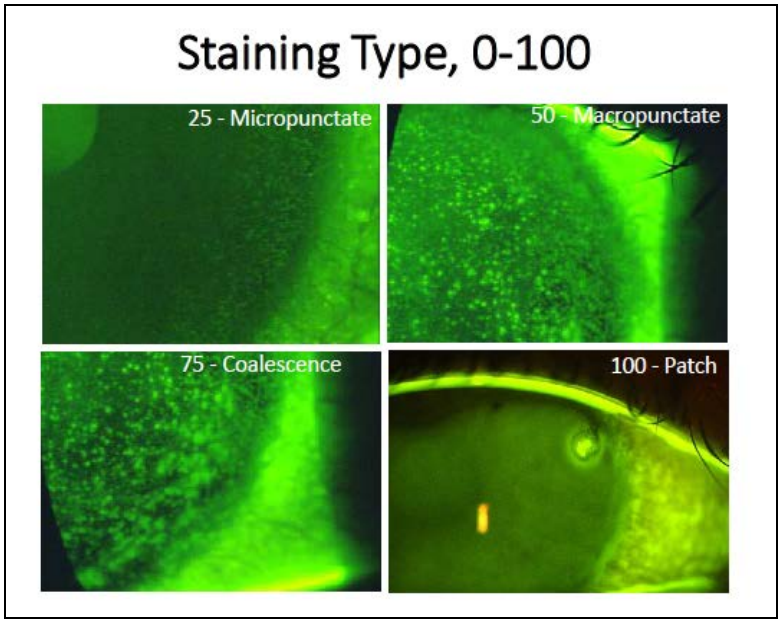
3.2 Methods

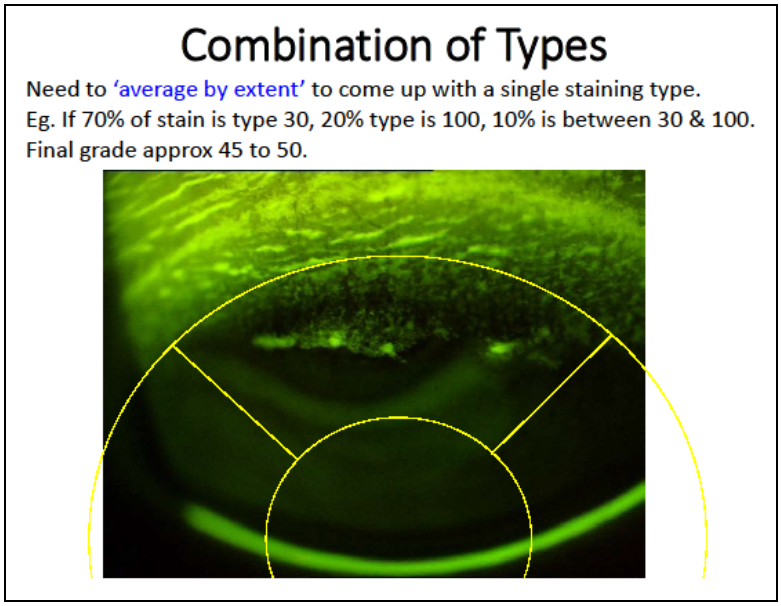
3.2.1 Images

Twenty-two slit-lamp biomicroscope images of corneal staining were sourced. Images were included if they had been captured using a blue filter over the illumination system and a yellow barrier filter in front of the observation system. This set-up of the slit-lamp biomicroscope optimises the view of corneal staining and is the appearance with which all observers are familiar with when grading corneal staining. The images were selected by observer O, with the specific intent to represent a spread across the entire 0-100 range of the *type* and the *extent* grades of the corneal staining. Any assessment of a scale should target the entire scale because measures of agreement calculated across just one section of a scale cannot be extrapolated to apply across the entire scale.[6,9] Additionally, the images had to be in clear focus and of sufficient illumination for the borders of the staining regions to be easily discernable.

All images were superimposed with a template of the five corneal zones and red arrows to indicate which zone should be graded. Only one zone per eye was graded because, due to the curvature of the cornea, it is often not possible to optimise the image of staining in all five zones in a single photograph. Additionally, in many images the lid or a light reflex impeded full view of specific zones, and therefore this zone selection further controlled the quality of the image in the specific zones being graded.

The images were collated into a PowerPoint presentation, which included three instructional slides at the beginning explaining how to use the scale, Figure 3-5.





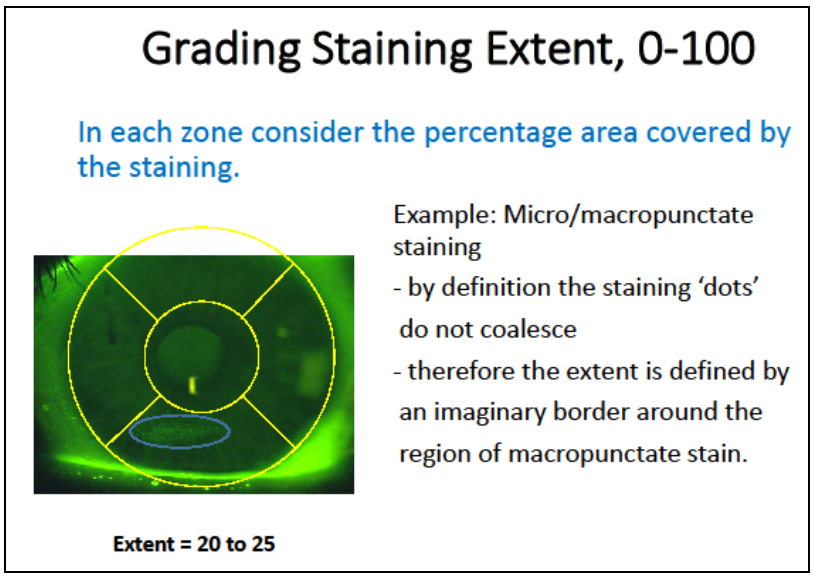


Figure 3-5: Instructions for the agreement experiment.

3.2.2 Observers

Fifteen observers (IDs A-O), participated in this agreement experiment. The observers were all aware of the CORE corneal staining scale, however, they had differing time periods of experience actually using it in clinical research. One observer (ID A) had not used the scale at all and had not received any previous training on its use. All other observers had received at least an initial training on the use of the scale. The number of years each observer had actively used the scale was recorded.

3.2.3 Grading sessions

A research assistant created the PowerPoint presentations, set up the computer and monitored all sessions to ensure the observer followed the instructions and that the session experiences were uniform. Consent was not required for this experiment because it was an internal training exercise as well as an assessment of the grading scale.

The observers attended two grading sessions, each scheduled at least one week apart. They recorded their grades for staining type and extent for all twenty-two images on a paper recording sheet which also provided some comments to aid the interpretation of the image, Figure 3-6. At both sessions the images were numbered sequentially from one to twenty-two, however the order was randomised and the order at Week-1 was not the same as Week-2.

Each grading session was held in the same room, on the same computer. All observers were allowed to self-regulate their time within each session and could review their grade and/or the instructions whenever needed. The grading session was scheduled to last 45-minutes to avoid anyone feeling they had to rush; most observers completed the session within 30-minutes.

Grading Sheet

CORE Corneal Staining Grading Scale – Week 1

Name: Date:

Image Number	Grading		
	Type (0-100)	Extent (0-100)	Comments for image
1			Assume no staining under top lid
2			-
3			Assume no staining under top lid
4			Ignore bulb reflection – assume no staining under that
5			Dark streak is simply tear layer thinning
6			Assume staining continues under top lid
7			-
8			-
9			Dark patch is simply tear layer thinning
10			-
11			Ignore bulb reflection – assume no staining under that
12			-
13			-
14			-
15			-
16			Ignore bulb reflection – assume no staining under that
17			-
18			-
19			-
20			Background streaks are not staining
21			Assume no staining under top lid
22			The glow is actually staining

Figure 3-6: Recording sheet for Week-1.

3.2.4 Analysis

INTER-OBSERVER AGREEMENT

The grades assigned by all the observers to the same image were compared for their agreement. This data will indicate whether certain observers grade higher or lower than others. This may also be termed inter-observer repeatability.

INTRA-OBSERVER AGREEMENT

All of the analyses and visualisation of the data for intra-observer agreement were carried out in the R-statistical software (version 3.5.3).[10]

The agreement of the grades assigned by each observer at the Week-1 and Week-2 sessions were evaluated in two ways:

i. Concordance;
ii. Tukey mean-difference plots.

The correlation coefficient of concordance (CCC) was calculated for each observer to assess concordance between grades across all images of Week-1 compared to Week-2.[11, 12] Perfect concordance would be indicated by a CCC value of 1.0 (i.e. their grading at Week-1 was identical to their grading at Week-2). The scale of the CCC is 0.0 - 1.0, where 0.0 indicates no agreement and 1.0 indicates perfect agreement. The CCC is the product of the Pearson correlation coefficient (r) and the agreement accuracy (χ_a) i.e. CCC = (r) x (χ_a). Similarly, r and χ_a are also scaled from 0.0 - 1.0, with the same end-scale descriptors.

Concordance data were determined using the AgreementInterval package of the R-statistical software.[13] Using these data, concordance plots for each observer between Week-1 versus Week-2 were created and are shown in Table 3-3 for the *type* data and Table 3-4 for the *extent* data. Each image from the grading scale is represented by one data point on the graph. Perfect concordance would be demonstrated when the grey, dashed best fit line corresponds exactly with the line of unity, the blue diagonal line. The corresponding numerical values for CCC, Pearson's r and accuracy (χ_a) are annotated on the plot.

In addition, the data per observer were plotted as Tukey mean-difference plots (also known as Bland-Altman plots).[14, 15] The Tukey mean-difference plots (hereafter referred to as 'Tukey plots') illustrate the difference between the grades from each week (Week-1 grade minus Week-2 grade) as a

function of the mean grade of both weeks (average of Week-1 and Week-2). In each graph, the mean of the differences between Week-1 and Week-2 grades, per image, is indicated by the grey, horizontal dashed line. A 95% confidence ellipse was determined for the data, based upon a multivariate normal distribution. This procedure determines the Euclidean distance from the centre of the data, for all data pairs. If the data were a circular cloud of data points (i.e. with little relationship between the mean and the difference), the ellipse would appear circular. In this experiment, as is generally the case for Tukey plots where the intention is to examine if the difference varies across the scale range, the data were dispersed along the x axis creating an ellipse. In those cases where the relationship between the dependent variable (mean grade) and the independent variable (the difference in grades) is constant, the ellipse will be oriented with the long axis parallel to the x-axis. Conversely, the relationship between dependent and independent variables may vary along the x-axis because the size and/or sign of the difference in measures depends on the magnitude of the mean. In such cases, the long axis of the ellipse would be tilted relative the x-axis. An ellipse was chosen rather than the more typical horizontal limits of agreement (1.96x standard deviation of the differences) because it appeared from Figure 3-7 and Figure 3-8 that agreement between weeks followed the latter example and varied as a function of the mean grade. If, for example, the ellipse tilts further from zero towards the high end of the scale, then agreement is reduced at that high end of the scale only. In the cases that give rise to a tilted ellipse, the typical horizontal limits of agreement would be artificially wider to capture the horizontal upper and lower limits of the tilted ellipse.

If the grades of an observer were in perfect agreement between Week-1 and Week-2 for all the images, the Tukey plot would show the mean difference line intercepting zero on the y-axis, and all data points would lie on this line for the full extent of the measurement scale. If an ellipse were fitted, it would be dimensionless in the y-axis and it would extend along the x-axis, also parallel to the x-axis i.e. without tilt. In such a case of perfect agreement, the typical horizontal limits of agreement would be coincident with the mean of differences line.

3.3 Results

In all graphs and tables the fifteen observers are identified by a letter code, from A to O. These letter codes were assigned to indicate the relative level of experience the observer had using the CORE corneal staining scale. Observer A had no experience and no prior training, whereas all other

investigators experience ranged from less than one year up to ten years for observers M, N and O (Table 3-5).

The twenty-two images were coded with an alpha-numeric code, based on the original source of the images.

3.3.1 Inter-observer agreement

The mean grades of the Week-1 and Week-2 grading sessions were calculated for each image, for each observer. These data per image were graphed to illustrate the inter-observer agreement for each image, both for the *type* data, Figure 3-7, and for the *extent* data, Figure 3-8. To aid interpretation of the data, the images were ordered along the x-axis from lowest to highest according to the mean grade from the entire observer group (i.e. the x-axis is ordinal) and the y-axis is continuous to represent the 0-100 scale.

The *type* graph, Figure 3-7, illustrates a typical spread of grades per image to be in the range of 35 to 40 units. This spread remains similar across most of the scale, with the obvious exception of the highest grade, where all observers graded either 90 or 100, a spread of just 10 units.

The *extent* graph, Figure 3-8, illustrates that the spread of grades varies as the mean grade increases up the y-axis. The first 8 images have mean extent grades below 20 and the observer data is spread over approximately 20 units. This inter-observer data spread increases as the mean grade increases, with the exception of image L1. In particular, images H1, W1, T1, K1, I1 and B2 all show poor inter-observer agreement with the data spread over more than half of the scale.

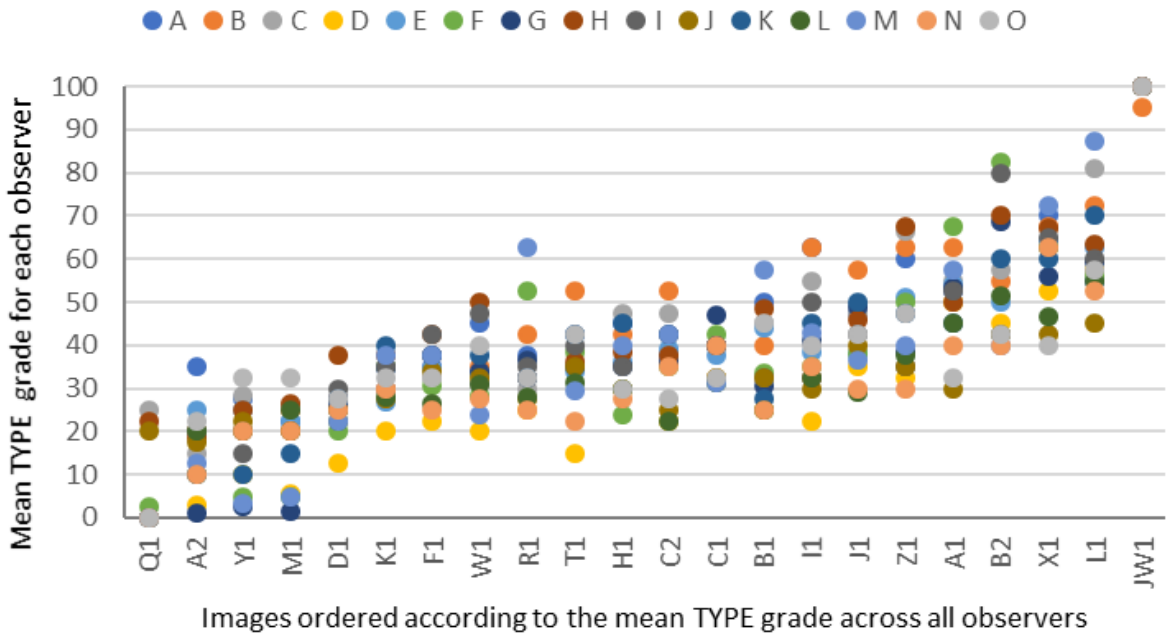


Figure 3-7: Mean (Week-1 and Week-2) TYPE grade per observer (observer A-O), by image. Images ordered along x-axis according to the group mean grade.

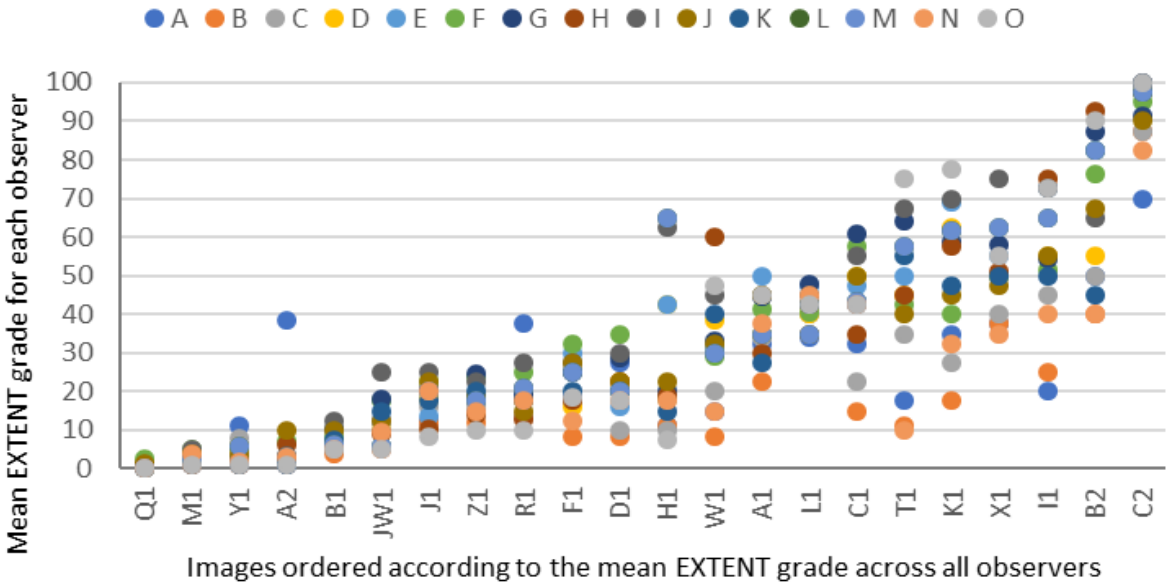


Figure 3-8: Mean (Week-1 and Week-2) EXTENT grade per observer (observer A-O), by image. Images ordered along x-axis according to the group mean grade.

The mean grades for all of the images combined, across both weeks were calculated for each observer, for type and extent, Figure 3-9. Graphing these data gave a visual indication of whether any particular observer tends to grade high or low. Across both graphs, the majority of the observers were within a 15-point spread for type, and a 10-point spread for extent. The graphs show that, on average, observer D tends to grade low for type, and observer I tends to grade high for extent.

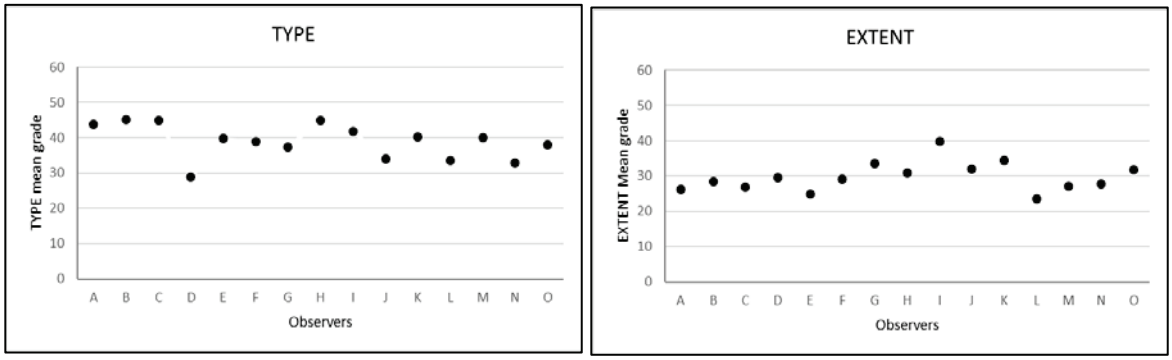
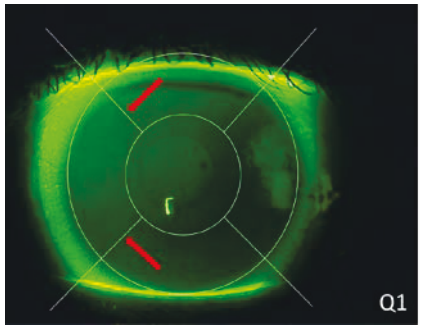


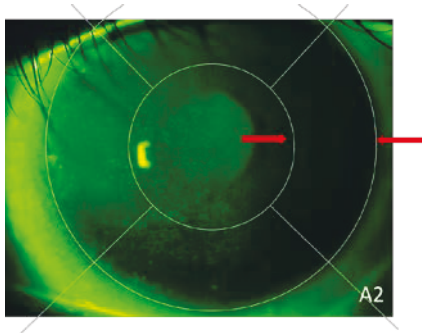
Figure 3-9: Mean grade for all images, both weeks, for each observer: TYPE data in graph on left, EXTENT data in graph on right.

The graphs in Figure 3-9 provide an indication of a trend to grade high or low, however, they do not provide information about whether this trend changes over the breadth of the scales, because all data is collapsed into one overall grade. The individual observer data for each image is presented in Table 3-1. This table facilitates a review of the data between observers for a single image. Also listed in this table are the grade differences between Week-1 and Week-2, as well as the mean of these differences, which was calculated from the absolute values of these differences i.e. ignoring which week offered the highest grade. The mean of the (absolute) differences provides information about the agreement of the group per image.

The images are ordered within Table 3-1 in ascending mean *type* grade.

Table 3-1: Data set for each image, ordered by ascending mean grade for type. * indicates absolute value

IMAGE	Obs	TYPE				EXTENT			
Q1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	0	0	0.0	0	0	0	0.0	0
	B	0	0	0.0	0	0	0	0.0	0
	C	25	25	25.0	0	1	0	0.5	1
	D	0	0	0.0	0	0	1	0.5	1
	E	0	0	0.0	0	0	0	0.0	0
	F	5	0	2.5	5	5	0	2.5	5
	G	0	0	0.0	0	0	0	0.0	0
	H	25	20	22.5	5	1	0	0.5	1
	I	0	0	0.0	0	0	0	0.0	0
	J	20	20	20.0	0	2	0	1.0	2
	K	0	0	0.0	0	0	0	0.0	0
	L	0	0	0.0	0	0	1	0.5	1
	M	0	0	0.0	0	0	0	0.0	0
	N	0	0	0.0	0	0	0	0.0	0
	O	0	0	0.0	0	0	1	0.5	1
	Mean	5.0	4.3	4.7	0.7	0.6	0.2	0.4	0.8

A2		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	10	60	35.0	50	2	1	1.5	1
	B	20	20	20.0	0	1	4	2.5	3
	C	5	25	15.0	20	1	75	38.0	74
	D	5	1	3.0	4	2	10	6.0	8
	E	25	25	25.0	0	3	1	2.0	2
	F	10	10	10.0	0	7	2	4.5	5
	G	1	1	1.0	0	1	1	1.0	0
	H	17	20	18.5	3	12	1	6.5	11
	I	20	20	20.0	0	2	1	1.5	1
	J	15	20	17.5	5	10	7	8.5	3
	K	10	10	10.0	0	1	5	3.0	4
	L	15	25	20.0	10	5	2	3.5	3
	M	15	10	12.5	5	2	3	2.5	1
	N	10	10	10.0	0	4	1	2.5	3
	O	25	20	22.5	5	1	1	1.0	0
	Mean	13.5	18.5	16.0	6.8	3.6	7.7	5.6	7.9

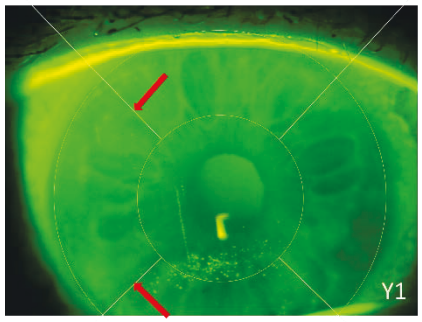
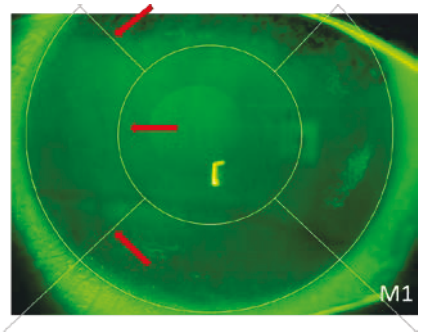
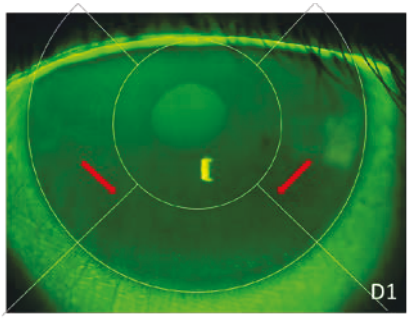
Y1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	25	30	27.5	5	2	10	6.0	8
	B	20	20	20.0	0	1	10	5.5	9
	C	25	32	28.5	7	1	20	10.5	19
	D	10	10	10.0	0	2	3	2.5	1
	E	25	25	25.0	0	1	1	1.0	0
	F	5	5	5.0	0	5	2	3.5	3
	G	1	4	2.5	3	1	2	1.5	1
	H	25	25	25.0	0	1	1	1.0	0
	I	10	20	15.0	10	1	2	1.5	1
	J	25	20	22.5	5	5	5	5.0	0
	K	10	10	10.0	0	1	5	3.0	4
	L	20	20	20.0	0	3	15	9.0	12
	M	5	2	3.5	3	2	3	2.5	1
	N	20	20	20.0	0	2	1	1.5	1
	O	35	30	32.5	5	1	1	1.0	0
	Mean	17.4	18.2	17.8	2.5	1.9	5.4	3.7	4.0

IMAGE	Obs	TYPE				EXTENT			
M1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	20	25	22.5	5	2	1	1.5	1
	B	25	25	25.0	0	1	0	0.5	1
	C	25	27	26.0	2	2	1	1.5	1
	D	10	1	5.5	9	2	3	2.5	1
	E	20	25	22.5	5	2	2	2.0	0
	F	5	5	5.0	0	5	5	5.0	0
	G	2	1	1.5	1	1	1	1.0	0
	H	28	25	26.5	3	1	2	1.5	1
	I	20	20	20.0	0	5	1	3.0	4
	J	20	20	20.0	0	5	5	5.0	0
	K	15	15	15.0	0	3	5	4.0	2
	L	20	30	25.0	10	3	4	3.5	1
	M	10	0	5.0	10	5	3	4.0	2
	N	20	20	20.0	0	3	1	2.0	2
	O	30	35	32.5	5	1	1	1.0	0
	Mean	18.0	18.3	18.1	3.3	2.7	2.3	2.5	1.1

D1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	20	35	27.5	15	20	25	22.5	5
	B	25	30	27.5	5	10	25	17.5	15
	C	25	25	25.0	0	10	35	22.5	25
	D	10	15	12.5	5	20	20	20.0	0
	E	25	25	25.0	0	15	7	11.0	8
	F	20	20	20.0	0	50	20	35.0	30
	G	26	26	26.0	0	30	17	23.5	13
	H	35	40	37.5	5	20	15	17.5	5
	I	30	30	30.0	0	35	27	31.0	8
	J	25	25	25.0	0	25	20	22.5	5
	K	30	25	27.5	5	25	25	25.0	0
	L	20	25	22.5	5	20	10	15.0	10
	M	25	20	22.5	5	15	15	15.0	0
	N	25	25	25.0	0	15	20	17.5	5
	O	25	30	27.5	5	15	25	20.0	10
	Mean	24.4	26.4	25.4	3.3	21.7	20.4	21.0	9.3

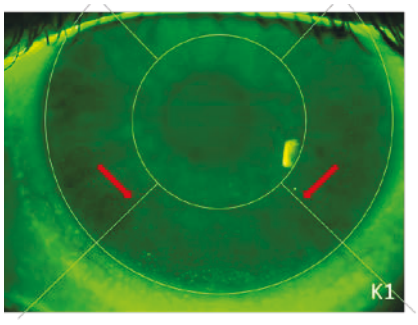
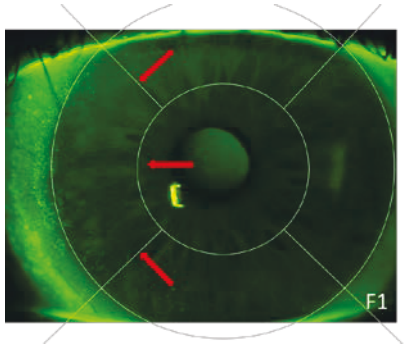
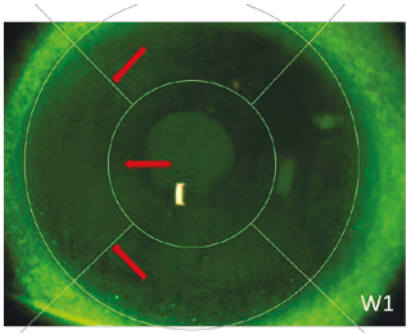
K1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	30	30	30.0	0	25	70	47.5	45
	B	30	30	30.0	0	20	78	49.0	58
	C	35	35	35.0	0	25	45	35.0	20
	D	25	15	20.0	10	55	50	52.5	5
	E	25	29	27.0	4	72	15	43.5	57
	F	35	35	35.0	0	35	40	37.5	5
	G	34	35	34.5	1	40	66	53.0	26
	H	40	35	37.5	5	70	45	57.5	25
	I	30	40	35.0	10	75	78	76.5	3
	J	30	25	27.5	5	40	45	42.5	5
	K	45	35	40.0	10	50	65	57.5	15
	L	28	28	28.0	0	25	30	27.5	5
	M	35	40	37.5	5	45	30	37.5	15
	N	25	35	30.0	10	25	80	52.5	55
	O	30	35	32.5	5	75	45	60.0	30
	Mean	31.8	32.1	32.0	4.3	45.1	52.1	48.6	24.6

IMAGE	Obs	TYPE				EXTENT			
F1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	20	50	35.0	30	10	15	12.5	5
	B	40	35	37.5	5	10	25	17.5	15
	C	30	35	32.5	5	20	28	24.0	8
	D	25	20	22.5	5	17	25	21.0	8
	E	32	37	34.5	5	25	7	16.0	18
	F	35	26	30.5	9	35	15	25.0	20
	G	38	37	37.5	1	28	35	31.5	7
	H	35	50	42.5	15	15	20	17.5	5
	I	45	40	42.5	5	25	22	23.5	3
	J	40	28	34.0	12	30	30	30.0	0
	K	40	35	37.5	5	20	30	25.0	10
	L	25	28	26.5	3	20	15	17.5	5
	M	40	35	37.5	5	25	10	17.5	15
	N	25	25	25.0	0	10	25	17.5	15
	O	35	30	32.5	5	12	20	16.0	8
	Mean	33.7	34.1	33.9	7.3	20.1	21.5	20.8	9.5

W1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	40	50	45.0	10	10	40	25.0	30
	B	40	30	35.0	10	10	35	22.5	25
	C	35	27	31.0	8	25	20	22.5	5
	D	20	20	20.0	0	37	30	33.5	7
	E	35	32	33.5	3	30	7	18.5	23
	F	27	30	28.5	3	33	15	24.0	18
	G	33	35	34.0	2	21	33	27.0	12
	H	45	55	50.0	10	60	50	55.0	10
	I	50	45	47.5	5	50	45	47.5	5
	J	35	30	32.5	5	35	25	30.0	10
	K	40	35	37.5	5	30	40	35.0	10
	L	32	30	31.0	2	12	15	13.5	3
	M	20	28	24.0	8	25	15	20.0	10
	N	30	25	27.5	5	15	45	30.0	30
	O	40	40	40.0	0	50	60	55.0	10
	Mean	34.8	34.1	34.5	5.1	29.5	31.7	30.6	13.9

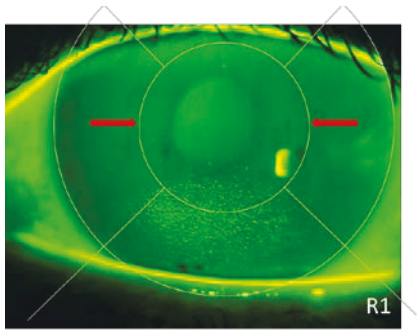
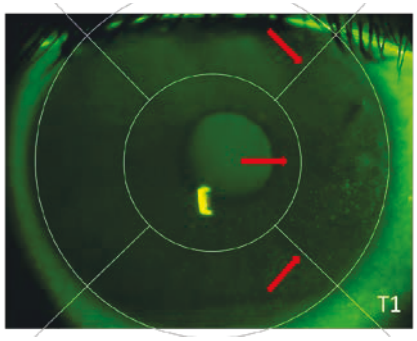
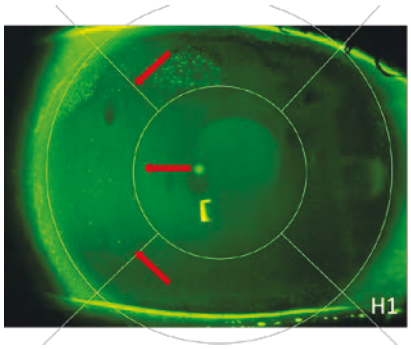
R1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	30	45	37.5	15	10	20	15.0	10
	B	55	30	42.5	25	15	25	20.0	10
	C	25	35	30.0	10	12	65	38.5	53
	D	30	20	25.0	10	15	15	15.0	0
	E	30	35	32.5	5	20	12	16.0	8
	F	50	55	52.5	5	28	20	24.0	8
	G	32	41	36.5	9	16	17	16.5	1
	H	30	35	32.5	5	11	15	13.0	4
	I	25	45	35.0	20	25	23	24.0	2
	J	30	25	27.5	5	15	22	18.5	7
	K	35	30	32.5	5	20	30	25.0	10
	L	28	28	28.0	0	12	15	13.5	3
	M	65	60	62.5	5	17	20	18.5	3
	N	25	25	25.0	0	15	10	12.5	5
	O	35	30	32.5	5	10	15	12.5	5
	Mean	35.0	35.9	35.5	8.3	16.1	21.6	18.8	8.6

IMAGE	Obs	TYPE				EXTENT			
T1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	25	60	42.5	35	15	50	32.5	35
	B	50	55	52.5	5	10	80	45.0	70
	C	40	30	35.0	10	50	20	35.0	30
	D	15	15	15.0	0	40	45	42.5	5
	E	35	33	34.0	2	50	12	31.0	38
	F	27	50	38.5	23	40	10	25.0	30
	G	29	41	35.0	12	41	50	45.5	9
	H	35	37	36.0	2	25	50	37.5	25
	I	40	40	40.0	0	70	87	78.5	17
	J	30	40	35.0	10	35	45	40.0	10
	K	45	40	42.5	5	60	65	62.5	5
	L	28	35	31.5	7	25	20	22.5	5
	M	27	32	29.5	5	35	30	32.5	5
	N	20	25	22.5	5	10	70	40.0	60
	O	40	45	42.5	5	80	65	72.5	15
	Mean	32.4	38.5	35.5	8.4	39.1	46.6	42.8	23.9

H1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	30	60	45.0	30	10	60	35.0	50
	B	30	55	42.5	25	12	75	43.5	63
	C	40	55	47.5	15	10	12	11.0	2
	D	35	25	30.0	10	25	20	22.5	5
	E	35	37	36.0	2	25	10	17.5	15
	F	28	20	24.0	8	20	20	20.0	0
	G	31	39	35.0	8	16	60	38.0	44
	H	32	45	38.5	13	28	15	21.5	13
	I	35	35	35.0	0	60	24	42.0	36
	J	30	30	30.0	0	25	16	20.5	9
	K	50	40	45.0	10	15	65	40.0	50
	L	30	30	30.0	0	20	10	15.0	10
	M	35	45	40.0	10	55	13	34.0	42
	N	25	30	27.5	5	15	8	11.5	7
	O	30	30	30.0	0	7	10	8.5	3
	Mean	33.1	38.4	35.7	9.1	22.9	27.9	25.4	23.3

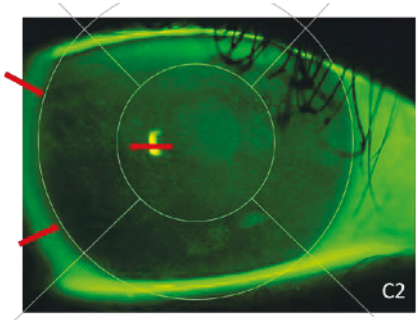
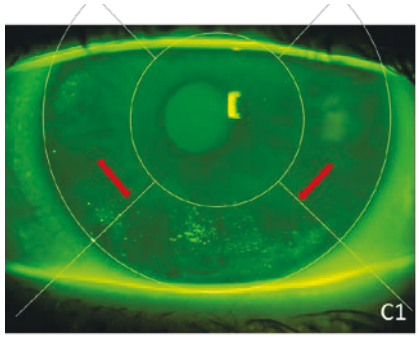
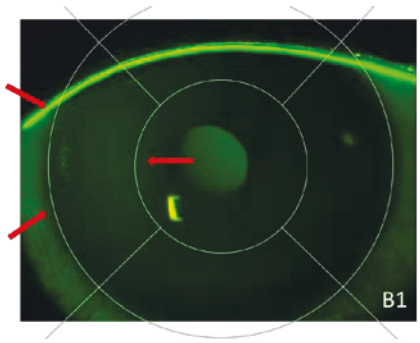
C2		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	25	50	37.5	25	50	100	75.0	50
	B	50	55	52.5	5	85	100	92.5	15
	C	40	55	47.5	15	80	90	85.0	10
	D	25	20	22.5	5	95	90	92.5	5
	E	38	40	39.0	2	100	90	95.0	10
	F	35	35	35.0	0	95	85	90.0	10
	G	33	39	36.0	6	93	100	96.5	7
	H	30	45	37.5	15	100	100	100.0	0
	I	35	50	42.5	15	100	90	95.0	10
	J	20	30	25.0	10	90	95	92.5	5
	K	35	50	42.5	15	100	95	97.5	5
	L	20	25	22.5	5	70	95	82.5	25
	M	30	55	42.5	25	95	90	92.5	5
	N	40	30	35.0	10	80	100	90.0	20
	O	25	30	27.5	5	100	100	100.0	0
	Mean	32.1	40.6	36.3	10.5	88.9	94.7	91.8	11.8

IMAGE	Obs	TYPE				EXTENT			
C1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	45	35	40.0	10	25	60	42.5	35
	B	30	35	32.5	5	15	37	26.0	22
	C	40	40	40.0	0	15	40	27.5	25
	D	25	40	32.5	15	40	45	42.5	5
	E	35	40	37.5	5	45	15	30.0	30
	F	55	30	42.5	25	55	50	52.5	5
	G	39	55	47.0	16	62	50	56.0	12
	H	40	40	40.0	0	30	50	40.0	20
	I	40	40	40.0	0	50	60	55.0	10
	J	30	35	32.5	5	55	60	57.5	5
	K	35	45	40.0	10	35	60	47.5	25
	L	28	35	31.5	7	35	30	32.5	5
	M	28	35	31.5	7	50	40	45.0	10
	N	45	35	40.0	10	35	50	42.5	15
	O	30	35	32.5	5	35	40	37.5	5
	Mean	36.3	38.3	37.3	8	38.8	45.8	42.3	15.3

B1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	40	60	50.0	20	6	5	5.5	1
	B	35	45	40.0	10	5	7	6.0	2
	C	45	45	45.0	0	5	5	5.0	0
	D	30	20	25.0	10	7	10	8.5	3
	E	50	38	44.0	12	7	3	5.0	4
	F	40	27	33.5	13	7	5	6.0	2
	G	33	28	30.5	5	10	7	8.5	3
	H	45	52	48.5	7	5	10	7.5	5
	I	35	30	32.5	5	5	11	8.0	6
	J	35	30	32.5	5	10	10	10.0	0
	K	30	25	27.5	5	5	20	12.5	15
	L	25	25	25.0	0	5	5	5.0	0
	M	65	50	57.5	15	6	5	5.5	1
	N	25	25	25.0	0	5	5	5.0	0
	O	50	40	45.0	10	5	5	5.0	0
	Mean	38.9	36.0	37.4	7.8	6.2	7.5	6.9	2.8

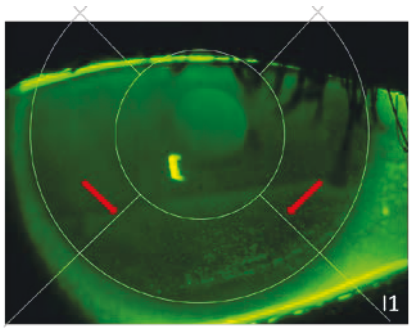
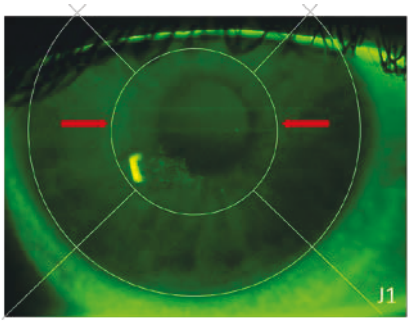
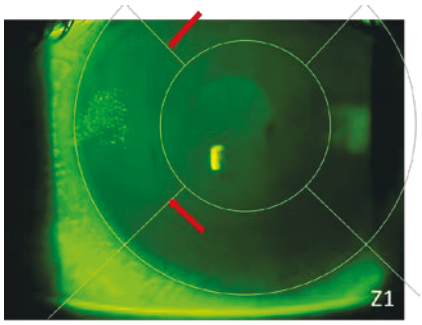
I1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	55	70	62.5	15	20	60	40.0	40
	B	60	65	62.5	5	25	70	47.5	45
	C	55	55	55.0	0	60	20	40.0	40
	D	25	20	22.5	5	50	60	55.0	10
	E	45	32	38.5	13	70	25	47.5	45
	F	35	35	35.0	0	38	40	39.0	2
	G	38	44	41.0	6	28	75	51.5	47
	H	40	25	32.5	15	80	40	60.0	40
	I	50	50	50.0	0	75	81	78.0	6
	J	30	30	30.0	0	50	65	57.5	15
	K	50	40	45.0	10	60	70	65.0	10
	L	30	35	32.5	5	35	30	32.5	5
	M	40	46	43.0	6	60	40	50.0	20
	N	30	40	35.0	10	40	75	57.5	35
	O	35	45	40.0	10	70	70	70.0	0
	Mean	41.2	42.1	41.7	6.7	50.7	54.7	52.7	24.0

IMAGE	Obs	TYPE				EXTENT			
J1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	40	55	47.5	15	10	20	15.0	10
	B	55	60	57.5	5	12	25	18.5	13
	C	45	50	47.5	5	12	15	13.5	3
	D	40	30	35.0	10	16	20	18.0	4
	E	40	36	38.0	4	12	12	12.0	0
	F	33	43	38.0	10	23	20	21.5	3
	G	41	57	49.0	16	20	15	17.5	5
	H	45	47	46.0	2	11	15	13.0	4
	I	40	45	42.5	5	20	21	20.5	1
	J	40	40	40.0	0	25	22	23.5	3
	K	60	40	50.0	20	20	30	25.0	10
	L	28	30	29.0	2	15	20	17.5	5
	M	35	38	36.5	3	15	20	17.5	5
	N	30	30	30.0	0	20	10	15.0	10
	O	50	35	42.5	15	7	10	8.5	3
	Mean	41.5	42.4	41.9	7.5	15.9	18.3	17.1	5.3

Z1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	60	60	60.0	0	10	20	15.0	10
	B	70	55	62.5	15	12	20	16.0	8
	C	65	68	66.5	3	15	20	17.5	5
	D	30	35	32.5	5	17	20	18.5	3
	E	65	37	51.0	28	22	12	17.0	10
	F	55	45	50.0	10	25	20	22.5	5
	G	31	46	38.5	15	25	15	20.0	10
	H	70	65	67.5	5	15	20	17.5	5
	I	40	30	35.0	10	20	24	22.0	4
	J	40	30	35.0	10	18	23	20.5	5
	K	55	40	47.5	15	20	25	22.5	5
	L	45	30	37.5	15	10	18	14.0	8
	M	35	45	40.0	10	15	15	15.0	0
	N	25	35	30.0	10	10	10	10.0	0
	O	55	40	47.5	15	10	13	11.5	3
	Mean	49.4	44.1	46.7	11.1	16.3	18.3	17.3	5.4

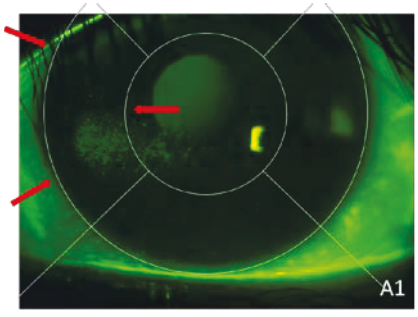
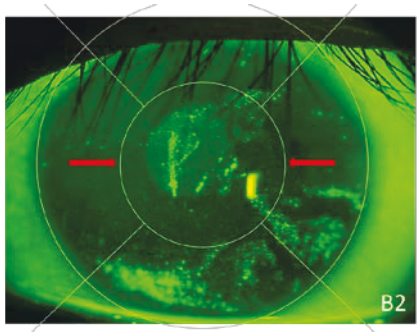
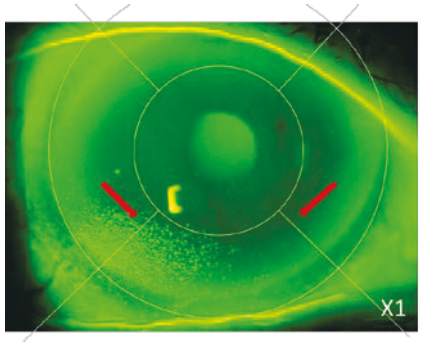
A1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	40	65	52.5	25	45	35	40.0	10
	B	55	70	62.5	15	25	40	32.5	15
	C	60	50	55.0	10	35	20	27.5	15
	D	40	50	45.0	10	35	50	42.5	15
	E	40	68	54.0	28	45	20	32.5	25
	F	75	60	67.5	15	38	40	39.0	2
	G	44	63	53.5	19	41	55	48.0	14
	H	35	65	50.0	30	40	30	35.0	10
	I	50	55	52.5	5	40	48	44.0	8
	J	30	30	30.0	0	40	45	42.5	5
	K	45	45	45.0	0	25	50	37.5	25
	L	45	45	45.0	0	35	33	34.0	2
	M	50	65	57.5	15	30	27	28.5	3
	N	30	50	40.0	20	35	50	42.5	15
	O	35	30	32.5	5	40	20	30.0	20
	Mean	44.9	54.1	49.5	13.1	36.6	37.5	37.1	12.3

IMAGE	Obs	TYPE				EXTENT			
B2		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	45	40	42.5	5	55	70	62.5	15
	B	45	65	55.0	20	45	80	62.5	35
	C	65	50	57.5	15	35	45	40.0	10
	D	40	50	45.0	10	40	65	52.5	25
	E	50	50	50.0	0	80	35	57.5	45
	F	90	75	82.5	15	83	40	61.5	43
	G	66	71	68.5	5	81	85	83.0	4
	H	70	70	70.0	0	95	50	72.5	45
	I	90	70	80.0	20	70	94	82.0	24
	J	45	40	42.5	5	70	70	70.0	0
	K	65	55	60.0	10	40	60	50.0	20
	L	53	50	51.5	3	50	65	57.5	15
	M	35	45	40.0	10	85	50	67.5	35
	N	45	35	40.0	10	40	90	65.0	50
	O	40	45	42.5	5	90	90	90.0	0
	Mean	56.3	54.1	55.2	8.9	63.9	65.9	64.9	24.4

X1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	60	80	70.0	20	25	50	37.5	25
	B	65	70	67.5	5	40	75	57.5	35
	C	65	60	62.5	5	40	50	45.0	10
	D	55	50	52.5	5	50	50	50.0	0
	E	60	68	64.0	8	50	35	42.5	15
	F	65	60	62.5	5	50	35	42.5	15
	G	46	66	56.0	20	51	60	55.5	9
	H	67	67	67.0	0	52	50	51.0	2
	I	80	50	65.0	30	80	65	72.5	15
	J	45	40	42.5	5	45	50	47.5	5
	K	60	60	60.0	0	50	70	60.0	20
	L	45	48	46.5	3	45	40	42.5	5
	M	80	65	72.5	15	50	47	48.5	3
	N	80	45	62.5	35	35	55	45.0	20
	O	30	50	40.0	20	55	50	52.5	5
	Mean	60.2	58.6	59.4	11.7	47.9	52.1	50.0	12.3

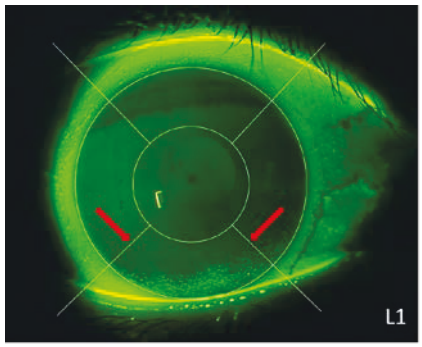
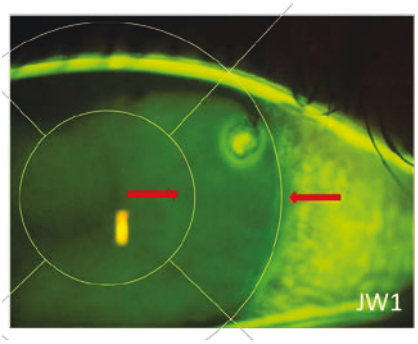
L1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	60	50	55.0	10	33	40	36.5	7
	B	75	70	72.5	5	30	40	35.0	10
	C	80	82	81.0	2	40	35	37.5	5
	D	50	65	57.5	15	40	40	40.0	0
	E	65	60	62.5	5	45	40	42.5	5
	F	55	58	56.5	3	48	45	46.5	3
	G	61	58	59.5	3	48	50	49.0	2
	H	72	55	63.5	17	45	40	42.5	5
	I	60	60	60.0	0	50	48	49.0	2
	J	50	40	45.0	10	45	33	39.0	12
	K	80	60	70.0	20	45	40	42.5	5
	L	55	55	55.0	0	55	40	47.5	15
	M	90	85	87.5	5	30	45	37.5	15
	N	50	55	52.5	5	45	45	45.0	0
	O	70	45	57.5	25	40	40	40.0	0
	Mean	64.9	59.9	62.4	8.3	42.6	41.4	42.0	5.7

IMAGE	Obs	TYPE				EXTENT			
JW1		Wk 1	Wk 2	Mean	Wk1-Wk2*	Wk 1	Wk 2	Mean	Wk1-Wk2*
	A	100	100	100.0	0	10	10	10.0	0
	B	90	100	95.0	10	5	7	6.0	2
	C	100	100	100.0	0	14	15	14.5	1
	D	100	100	100.0	0	10	10	10.0	0
	E	100	100	100.0	0	11	5	8.0	6
	F	100	100	100.0	0	15	12	13.5	3
	G	100	100	100.0	0	18	8	13.0	10
	H	100	100	100.0	0	8	15	11.5	7
	I	100	100	100.0	0	25	18	21.5	7
	J	100	100	100.0	0	15	20	17.5	5
	K	100	100	100.0	0	15	25	20.0	10
	L	100	100	100.0	0	7	10	8.5	3
	M	100	100	100.0	0	5	7	6.0	2
	N	100	100	100.0	0	7	5	6.0	2
	O	100	100	100.0	0	5	10	7.5	5
	Mean	99.3	100.0	99.7	0.7	11.3	11.8	11.6	4.2

** the values are converted to absolute values*

The mean of all grades from all observers is shown in Table 3-2, together with the mean standard deviation and the 95% confidence interval, calculated as 1.96 x the mean standard deviation. The confidence interval is the range, within which it is 95% certain that the true mean exists.

Table 3-2: For each image: mean grade, mean standard deviation (StDev), 95% confidence interval (CI).

	TYPE			EXTENT		
Image	**Mean of Obs mean grades**	**Mean of StDev**	**95% CI**	**Mean of Obs mean grades**	**Mean of StDev**	**95% CI**
Q1	4.7	9.3	18.2	0.4	0.7	1.4
A2	16.0	8.9	17.4	5.6	9.2	18.0
Y1	17.8	9.6	18.8	3.7	3.0	5.9
M1	18.1	9.5	18.6	2.5	1.5	2.9
D1	25.4	5.3	10.4	21.0	6.2	12.2
K1	32.0	5.2	10.2	48.6	12.1	23.7
F1	33.9	5.9	11.6	20.8	5.5	10.8
W1	34.5	8.4	16.5	30.6	12.7	24.9
R1	35.5	10.3	20.2	18.8	6.9	13.5
T1	35.5	8.9	17.4	42.8	16.3	31.9
H1	35.7	7.2	14.1	25.4	12.2	23.9
C2	36.3	8.9	17.4	91.8	6.7	13.1
C1	37.3	4.8	9.4	42.3	10.3	20.2
B1	38.9	10.8	21.2	6.9	2.3	4.5
I1	41.7	11.7	22.9	52.7	12.5	24,5
J1	41.9	7.8	15.3	17.1	4.4	8.6
A1	48.5	10.2	20.0	37.1	6.2	12.2
Z1	46.7	12.6	24.7	17.3	3.8	7.4
B2	55.2	14.3	28.0	64.9	13.3	26.1
X1	59.4	9.9	19.4	50.0	8.8	17.2
L1	62.4	11.2	22.0	42.0	4.6	9.0
JW1	99.7	1.3	2.5	11.8	5.8	11.4

3.3.2 Intra-observer agreement

For each observer, two different graphs were plotted to demonstrate different aspects of the intra-observer agreement; concordance plots and Tukey plots. These were collated into separate tables, one to illustrate the agreement for the *type* data, Table 3-3, and another for the *extent* data, Table 3-4. In

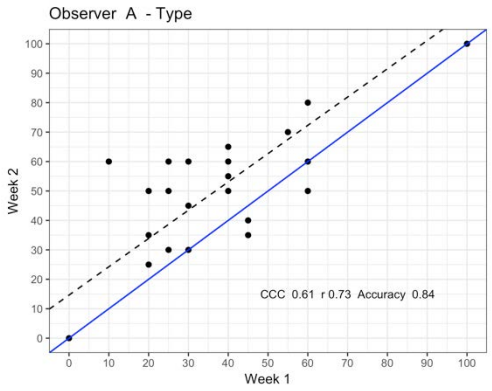
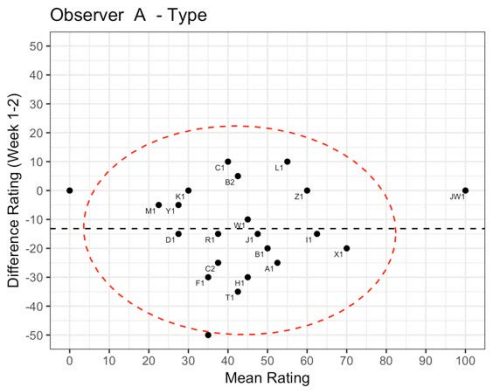
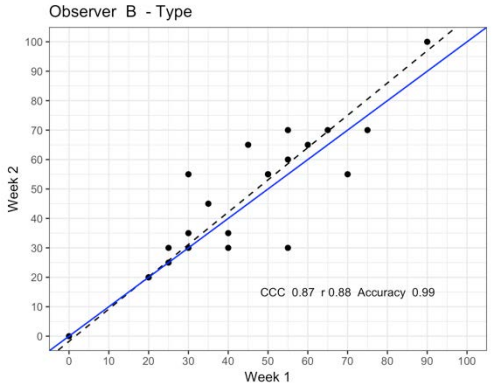
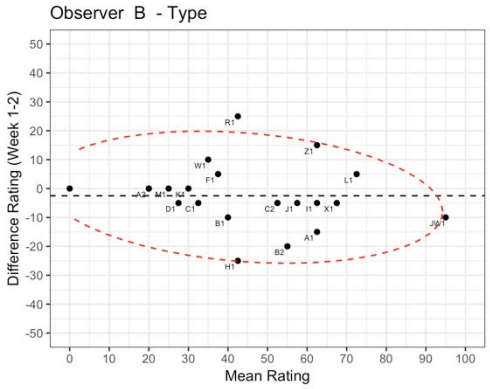
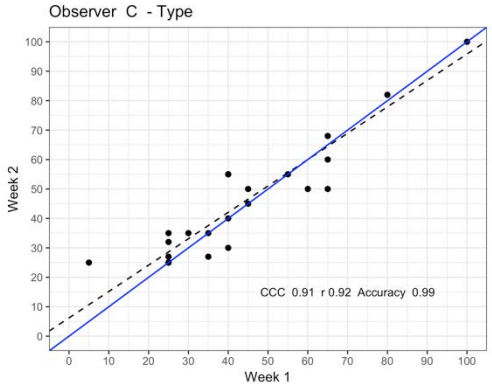
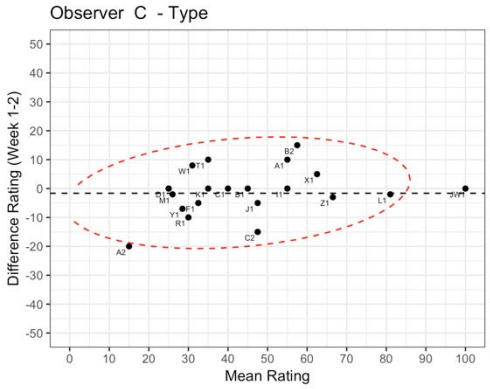
each table, the observers are ordered according to their years of experience with the scale, with the least experienced, observer A, listed first.

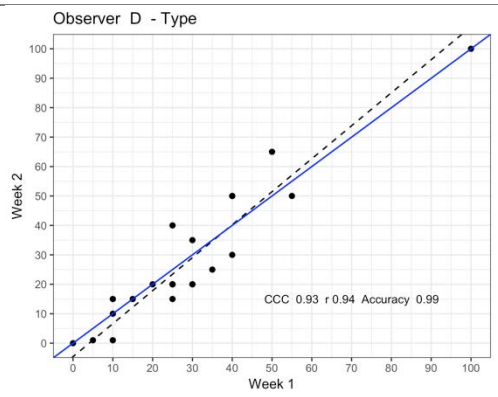
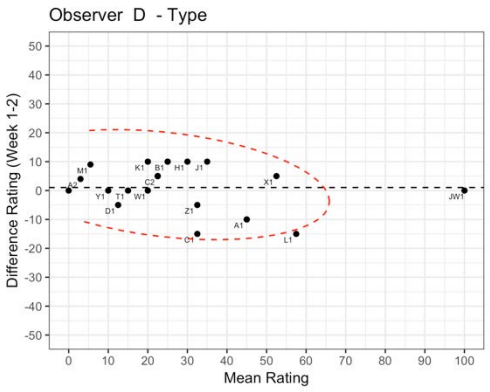
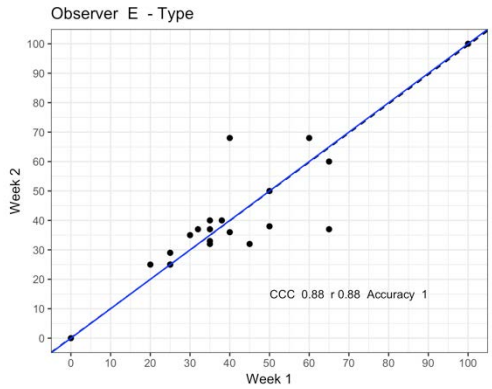
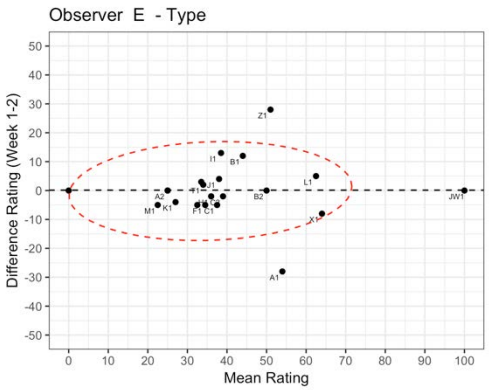
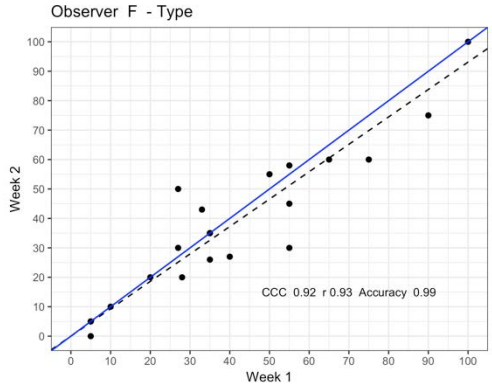
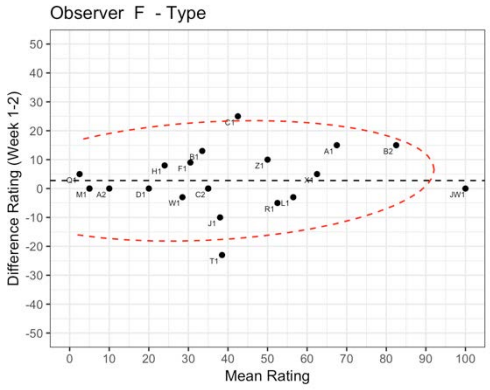
The concordance plots show the Week-2 data as a function of Week-1 data. Each image is represented by one data point on the graph. Perfect repeatability would be demonstrated if all data points lay on the blue diagonal line, the line of unity, which is the line of zero difference between grades from Week-1 and Week-2. These plots also display the values for the correlation coefficient of concordance (CCC), Pearson's correlation coefficient (r) and accuracy (χ_a) for each observer, also in Table 3-5. Perfect agreement between the grades for Week-1 and Week-2 would lead to all three of these indices having a value of 1.0. In each concordance plot, if the best-fit data line is coincident with the line of unity, then accuracy is 1.0. Pearson's r describes how closely the data points can be fit to a line. The product of accuracy and Pearson's r provides the CCC value.

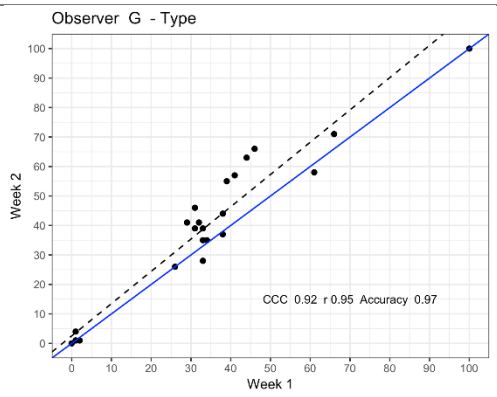
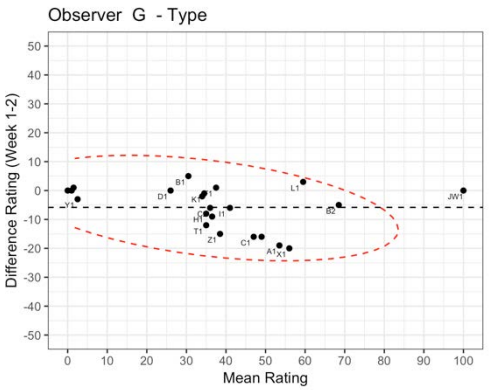
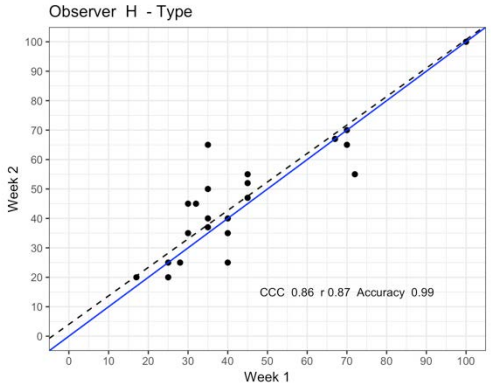
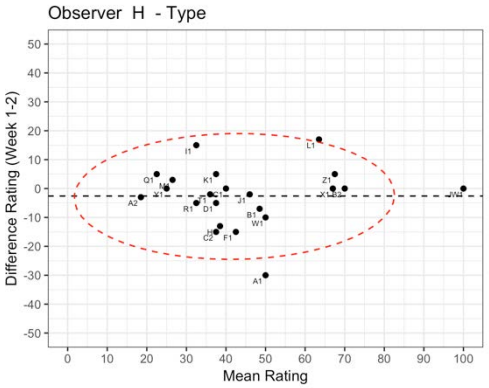
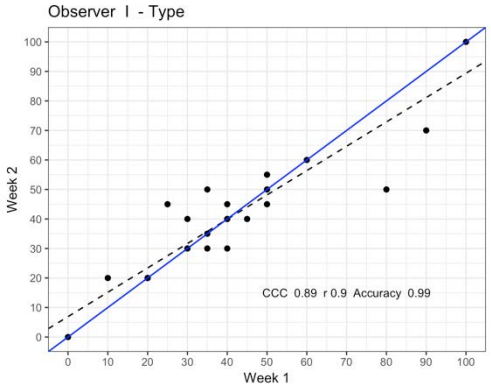
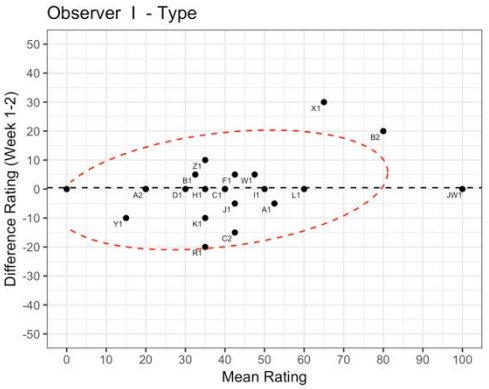
The *extent* grade concordance plots for observers J and K illustrate the different meanings of these indices. Both have accuracy of 1.0, however, the data points of observer J are a closer fit to the line, leading to observer J having a higher value for Pearson's r (0.97 versus 0.94) and thus to a higher CCC value (0.97 versus 0.94). The high accuracy is reflected in the Tukey plots by the mean line being almost coincident with zero on the y-axis. The poorer line-fit of the data from observer K is reflected in the greater vertical spread of the ellipse.

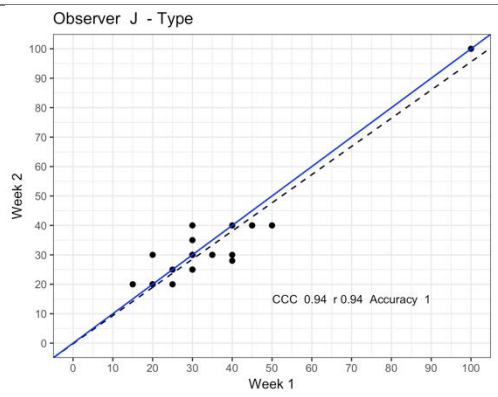
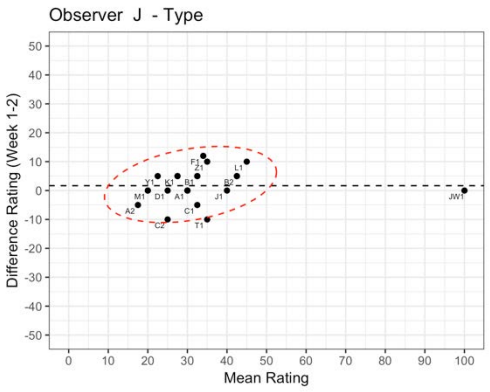
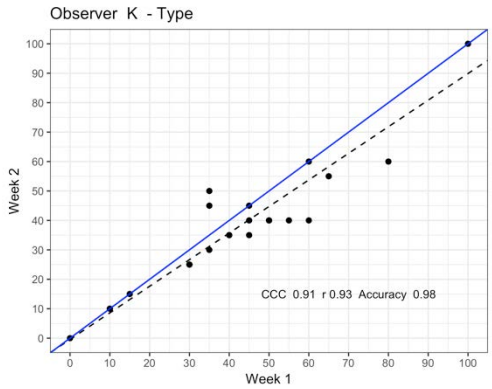
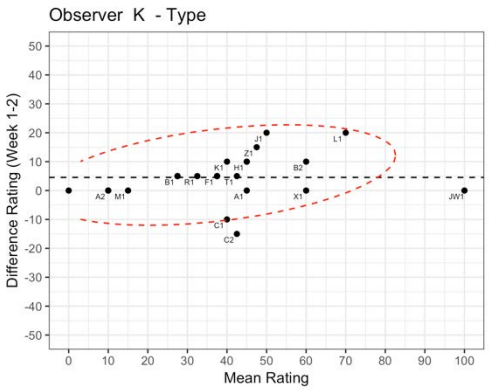
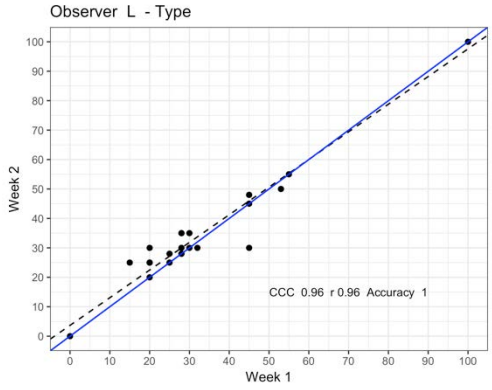
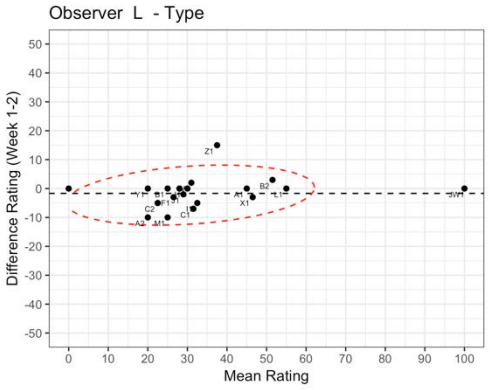
Observer A is the observer with no previous experience or training with the scale. Their concordance plot for type shows a data line that is almost parallel to the line of perfect unity, because the Week-2 grades were more commonly higher than Week-1. However, the data points are a poor fit to a line ($r = 0.73$) and, therefore, the Tukey plot resembles a circle, rather than an ellipse, indicating wide limits of agreement (poor agreement) along the entire the scale (x-axis).

Table 3-3: Staining TYPE by observer, ordered by years of experience: concordance graphs and Tukey plots with elliptical limits of agreement. Obs: observer; Exp: number of years of experience with the CORE staining scale; CCC: concordance correlation coefficient; r: Pearson's correlation coefficient; Accuracy: chi, χ_a.

Obs, Exp	Concordance	Tukey
Obs A **0yr**		
Obs B **<1yr**		
Obs C **<1yr**		

Obs, Exp	Concordance	Tukey
Obs D 1yr		
Obs E 1yr		
Obs F 2yr		

Obs, Exp	Concordance	Tukey
Obs G 3yr		
Obs H 5yr		
Obs I 6yr		

Obs, Exp	Concordance	Tukey
Obs J **7yr**		
Obs K **8yr**		
Obs L **9yr**		

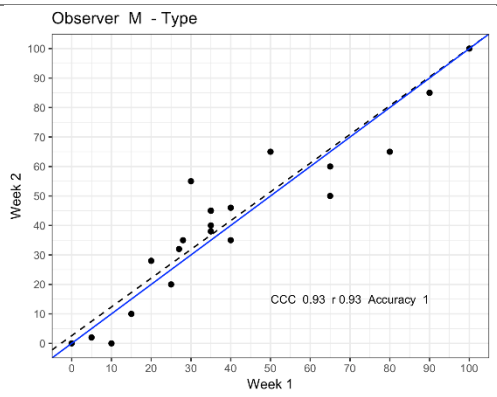
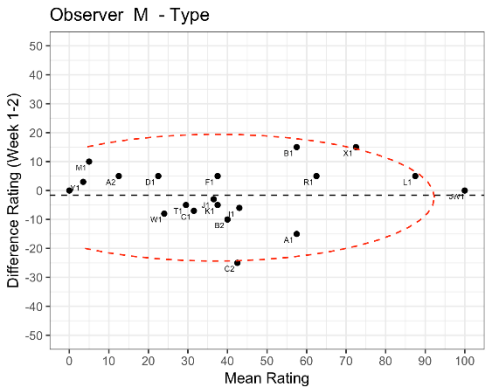
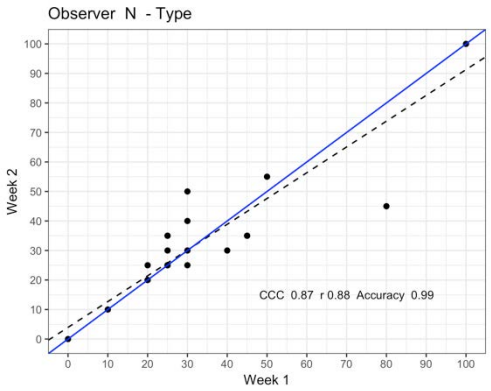
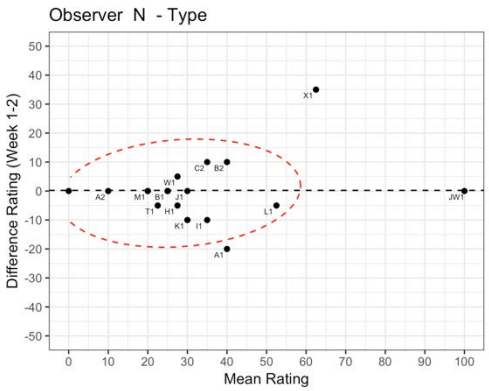
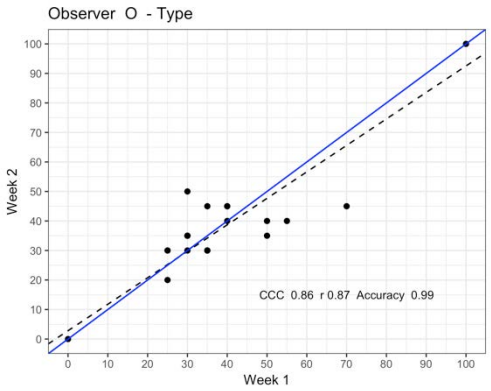
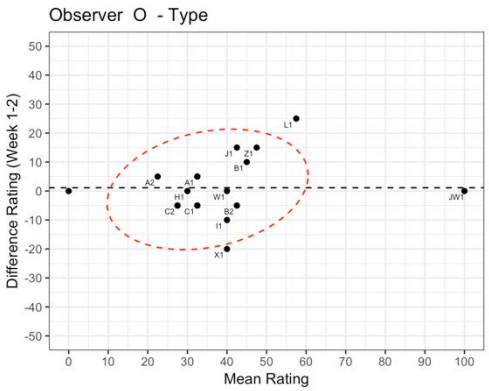
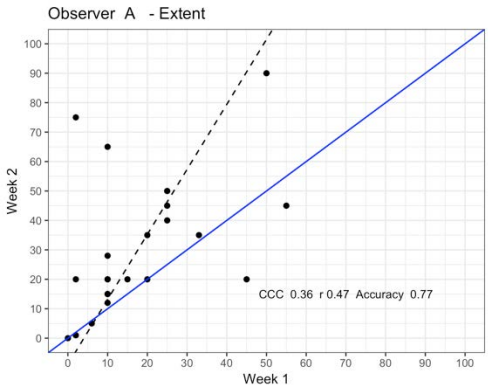
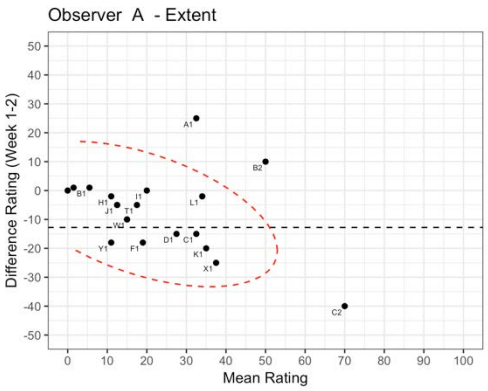
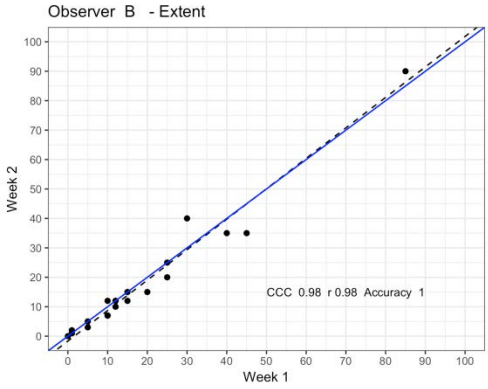
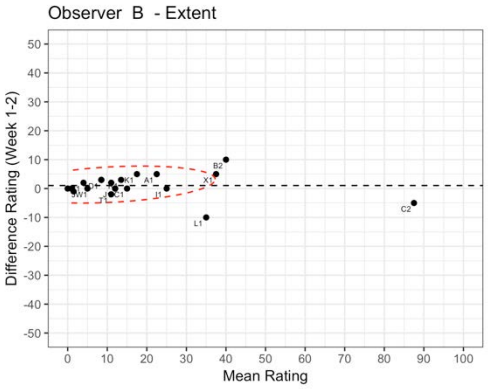
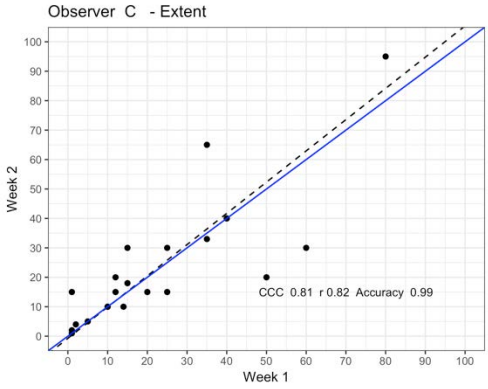
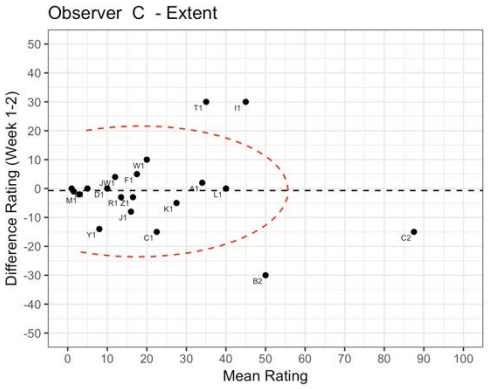
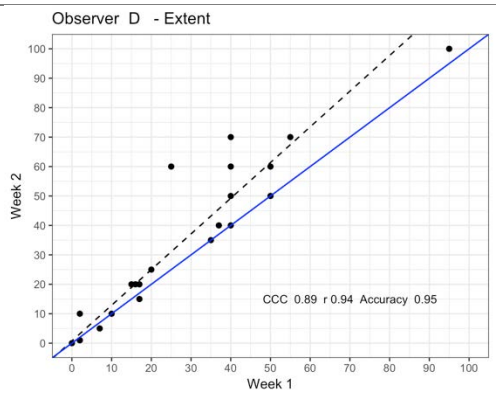
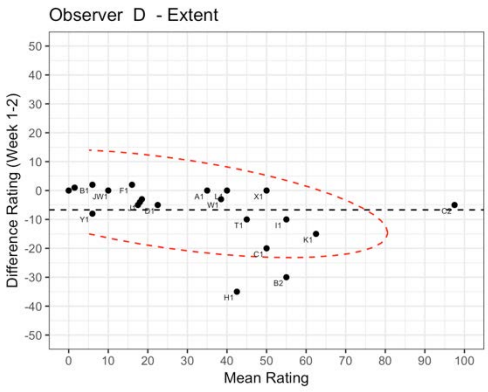
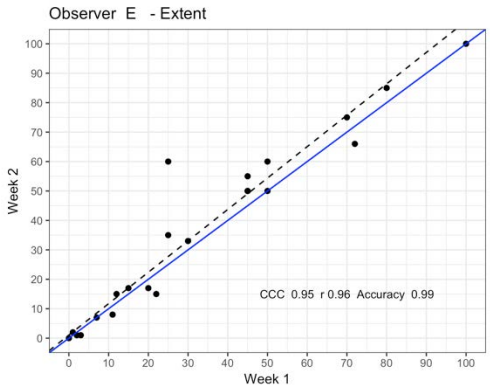
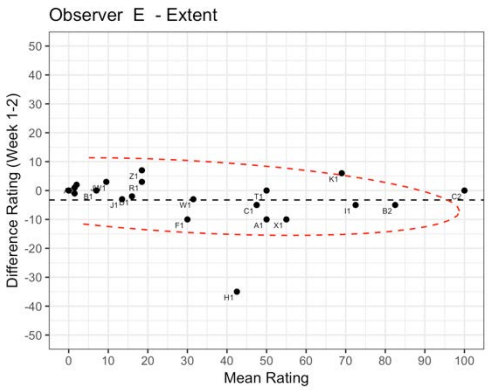
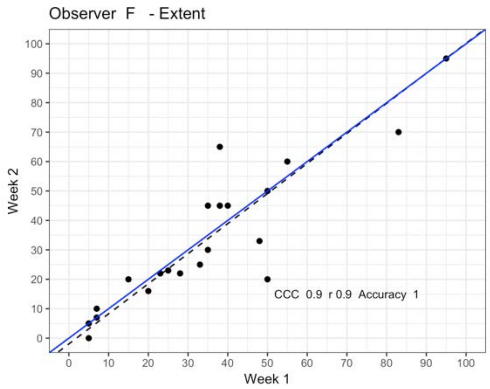
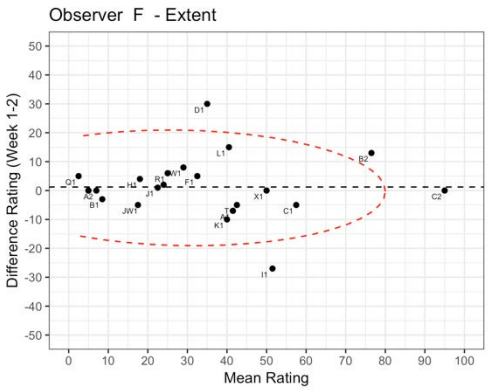
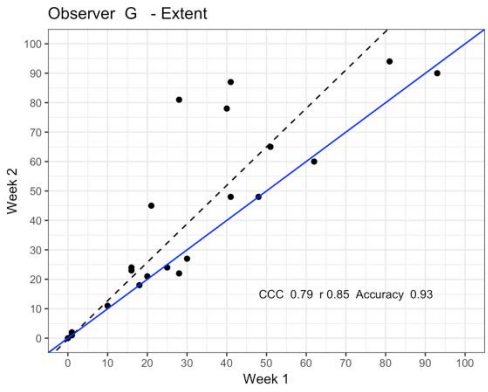
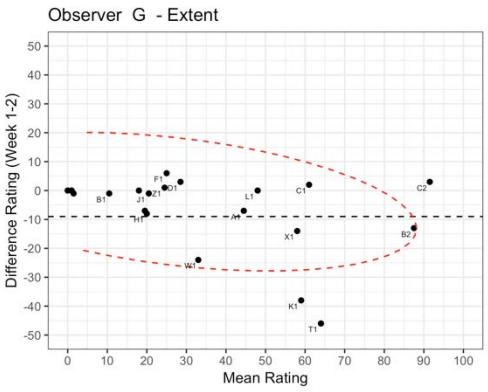
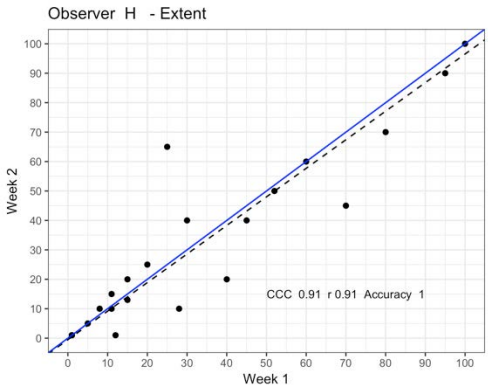
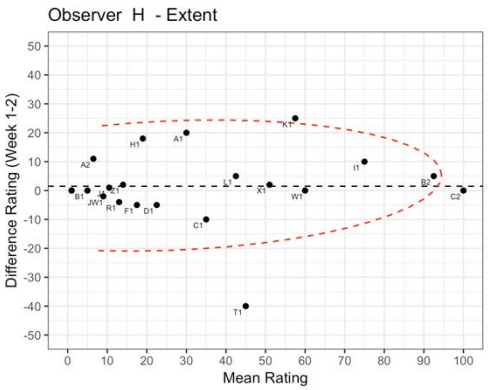
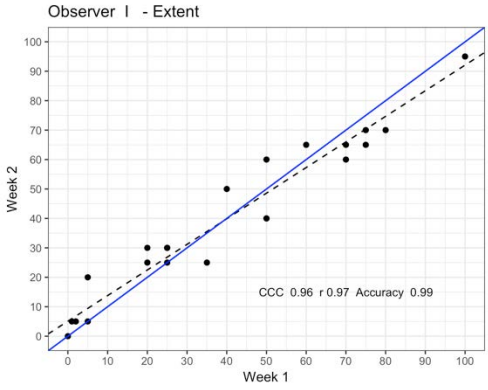
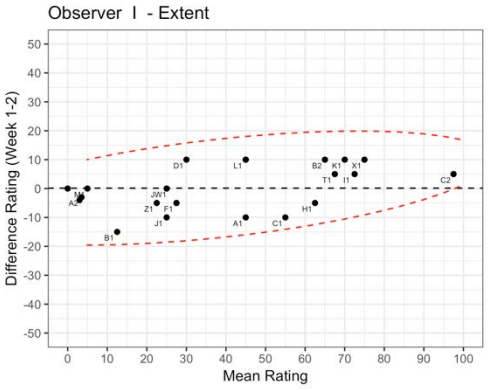
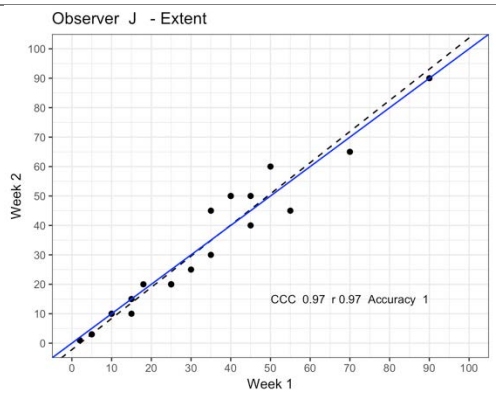
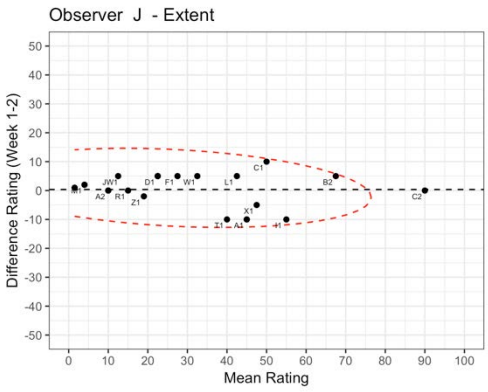
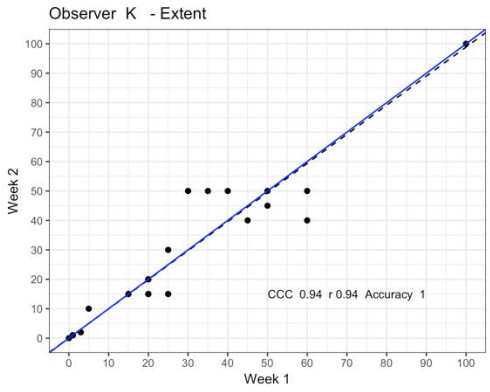
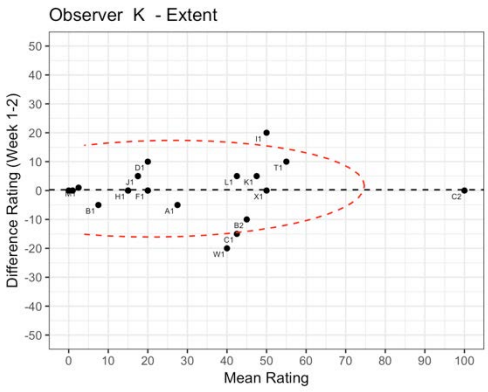
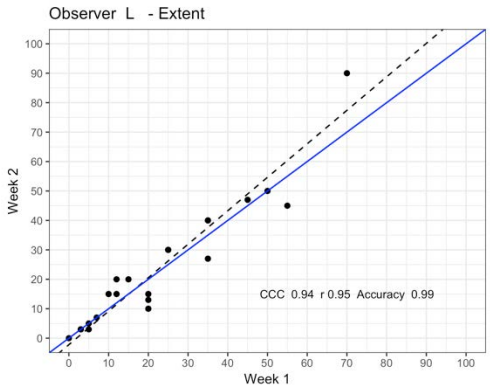
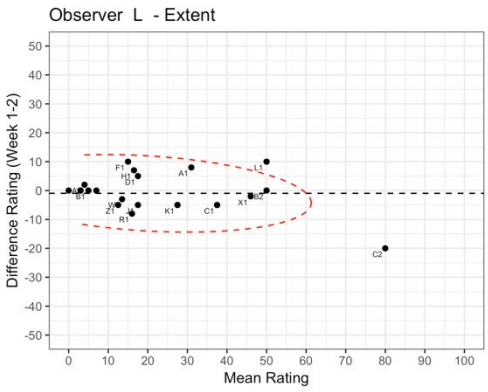
Obs, Exp	Concordance	Tukey
Obs M 10yr		
Obs N 10yr		
Obs O 10yr		

Table 3-4: Staining EXTENT by observer, ordered by years of experience: concordance graphs and Tukey plots with elliptical limits of agreement. Obs: observer; Exp: number of years of experience with the CORE staining scale; CCC: concordance correlation coefficient; r: Pearson's correlation coefficient; Accuracy: chi, χ_a.

Obs, Exp	Concordance	Tukey
Obs A **0yr**		
Obs B **<1yr**		
Obs C **<1yr**		

Obs, Exp	Concordance	Tukey
Obs D 1yr		
Obs E 1yr		
Obs F 2yr		

Obs, Exp	Concordance	Tukey
Obs G **3yr**		
Obs H **5yr**		
Obs I **6yr**		

Obs, Exp	Concordance	Tukey
Obs J **7yr**		
Obs K **8yr**		
Obs L **9yr**		

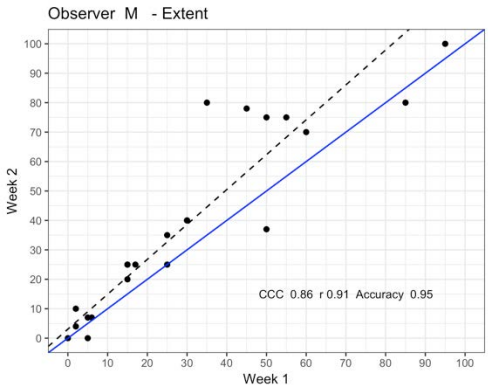
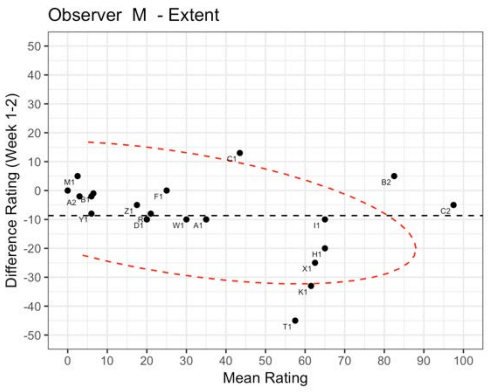
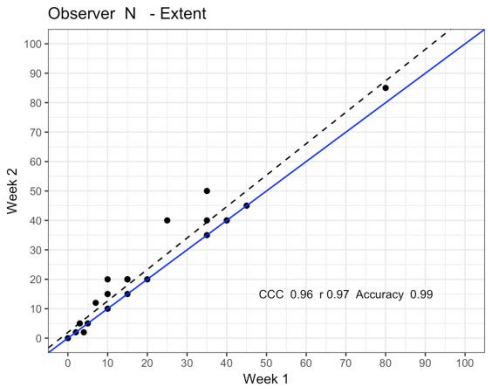
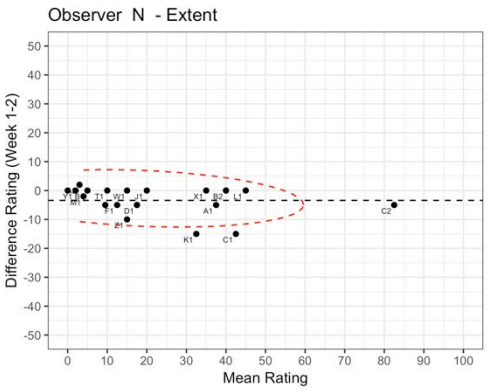
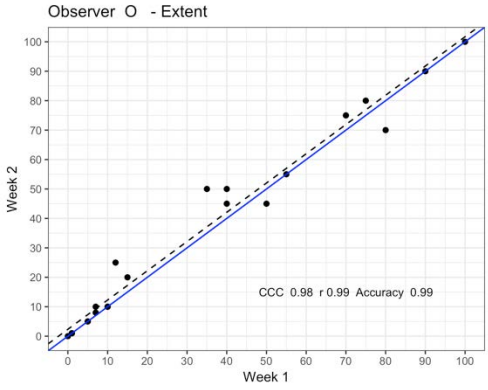
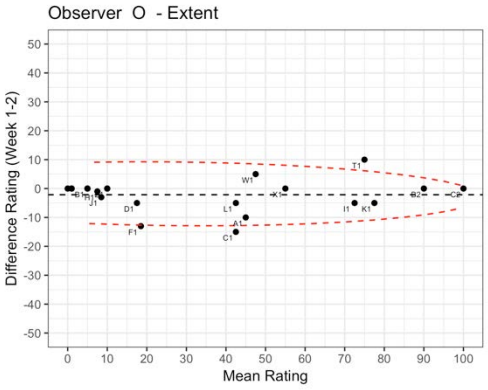
Obs, Exp	Concordance	Tukey
Obs M 10yr		
Obs N 10yr		
Obs O 10yr		

Table 3-5: Concordance indices for each observer; observers ordered by years of experience.

		TYPE			EXTENT		
Obs	**Exp**	**CCC**	**r**	χ_a	**CCC**	**r**	χ_a
A	0	0.61	0.73	0.84	0.36	0.47	0.77
B	<1	0.87	0.88	0.99	0.98	0.98	1.0
C	<1	0.91	0.92	0.99	0.81	0.82	0.99
D	1	0.93	0.94	0.99	0.89	0.94	0.95
E	1	0.88	0.88	1.00	0.95	0.96	0.99
F	2	0.92	0.93	0.99	0.90	0.90	1.00
G	3	0.92	0.95	0.97	0.79	0.85	0.93
H	5	0.86	0.87	0.99	0.91	0.91	1.00
I	6	0.89	0.90	0.99	0.96	0.97	0.99
J	7	0.94	0.94	1.00	0.97	0.97	1.00
K	8	0.91	0.93	0.98	0.94	0.94	1.00
L	9	0.96	0.96	1.00	0.94	0.95	0.99
M	10	0.93	0.93	1.00	0.86	0.91	0.95
N	10	0.87	0.88	0.99	0.96	0.97	0.99
O	10	0.86	0.87	0.99	0.98	0.99	0.99

3.3.3 Use of the steps of the scale

Histograms of all the grades assigned across all images, observers and weeks provides evidence that the 0-100 integer scales for type (Figure 3-10) and extent (Figure 3-11) were not used as true 101-step scales for selection of images used. There was over sampling of the numbers in steps of 5. The type grades were used as integers mainly within the range from 25 to 40, with limited use outside this range. The extent grades were commonly used as integers within the 0-15 range, with some use up to 35, and limited use for grades higher than 35.

Observer O selected images that covered the range of scale. The histsograms show that the full range was indeed covered. The type grades allocated by the observers appear normally distributed

around the grade 30. The extent grades used have a flatter distribution, which is biased towards the lower end of the scale.

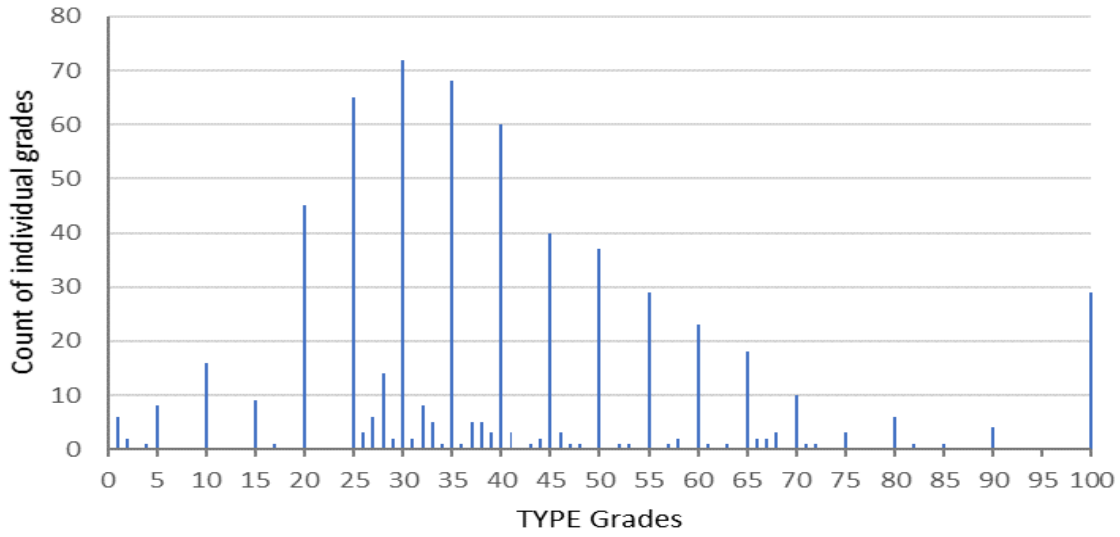


Figure 3-10: Count of the individual TYPE grades used.

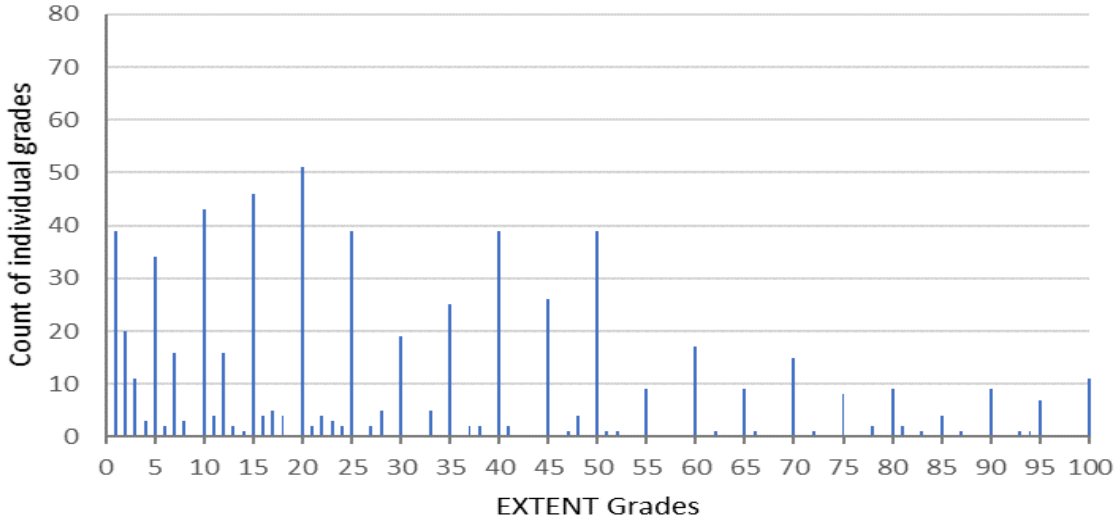


Figure 3-11: Count of the individual EXTENT grades used.

3.4 Discussion

The images provided a spread of type and extent grades that spanned the entire scales, although both show unequal distribution across the scales with bias towards the lower half. The lower to middle ranges reflect the level of staining appearance that observers most commonly deal with in clinical research and it is within this range of the scale where good agreement is most required in order to identify differences between products and/or treatments. The high regions of both scales often relate to an adverse response, and would thus typically be excluded from the comparison statistical analysis.

The inter-observer agreement was not good for either scale factors. The type grade exhibited a data spread of close to 40 units across the entire range of the scale. The extent grades showed closer inter-observer agreement at the lower end of the scale, within 20 units, but the agreement reduced as the extent increased. Poor inter-observer agreement for ocular grading scales has been reported previously[5, 16] and it is possible that more frequent training would improve this agreement. It is generally accepted that all grading scales require initial training to establish optimal grading techniques, as well as periodic retraining to avoid the observers redefining their internal references over time. Observer A was the only observer who had not received prior training and the fact that their intra-agreement was by far the lowest provides evidence that the training provided to the other observers was somewhat effective.

The results from observer A remain in the data presented even though there is argument for removing this data from the calculations of means due to the obvious lack of intra-observer agreement. Similarly, there are several data points that are significant outliers and appear to be blatant errors. For example, observer C recorded the extent for image A2 as grade 1 in Week-1 and 75 in Week-2, when the observer group mean was approximately 6. Likewise, observer B recorded the extent for image H1 as grade 12 in Week-2 and 75 in Week-2, when the observer group mean was approximately 25. All obvious outliers were confirmed with the source data, however it is most likely that there was an error either in recording the grade against the wrong image on the recording sheet, or perhaps grading the wrong zone of the image, despite the red arrows highlighting which zone to grade. Leaving these various suspect data in the tables will skew the mean values. The impact of these suspect data can be reduced by calculating the means with a ‘bootstrapping’ method.[17] This method provides a better approximation of the true mean by adjusting for the sample bias, thus reducing the impact of outliers. Obtaining a truer estimate of the mean grade will be valuable for future training as these means can be used to guide image selection. The distribution of the bootstrapped estimates can

be examined for normality; those distributions that are normally distributed around the mean grade can be classified as ‘good’ and those images that depart from normality (skewness, kurtosis or high standard error) as ‘bad’. The ‘good’ images can then be selected as training images and/or used as anchors for the scale at their mean grade. The ‘bad’ images may be valuable for discussion during group training sessions in order to establish a more uniform grading approach across all observers.

The concordance graphs illustrate how closely each observer graded in Week-2 compared to Week-1. With the exception of observer A, the CCC values are generally close to or above 0.90 (i.e. with moderate or better agreement). When viewing these concordance graphs, it is of value to review the Tukey plot alongside it, because this plot illustrates how the agreement varied along the scale by the tilt of the ellipse, and it also illustrates how much of the scale was utilised by the observer across all images. For example the extent Tukey plots of observers B and C show that their grades were clustered below 50. It could be argued that good agreement is easier to achieve when using a smaller part of the scale, however there is a wide difference between the CCC of observers B and C, 0.98 versus 0.81.

The results of this agreement experiment can be used to inform future training. Some images are associated with high observer agreement, as indicated by the mean of the absolute differences between the Week-1 and Week-2 grades. A low value of mean difference identifies the images of highest inter-observer agreement. Ideally, the images would demonstrate a low mean absolute difference grade for both *type* and *extent*. Examples of such images include image Q1 (type 0.7, extent 0.8), image M1 (type 3.3, extent 1.1) and image JW1 (type 0.7, extent 4.2). These mean absolute difference values may also be smaller when obvious outliers are removed, as explained earlier.

Establishing a reference grade per image can be a helpful strategy to guide the use of the scale and future agreement experiments because each observers’ grades would be compared to the reference grade. Another approach is to elect one observer to be the ‘gold standard’ observer and compare all other observers to this one person. The gold standard observer would be chosen as the person who shows the best concordance across the two weeks and who is consistently closest to the group mean. It is likely to be more difficult to identify one observer who is consistently closest to the mean across the entire range of both grading factors and hence the former method of determining standardised images may be more practical.

One more point to consider in the development of future training sessions is the difficulty of grading a static image when in practice this grading is conducted under 'live' conditions i.e. when the illumination can be adjusted, and the eye can blink to spread the tear film more evenly. The existence of large differences between 'live' grading and photographic grading of corneal staining has previously been reported by Sorbara *et al.*[18] who showed that photographic grading under-reported the staining level. There are assumptions made when grading a photograph that can be eliminated in the live scenario. For example, a strand of mucus in the tear film that is blinked away during live grading would be ignored, rather than being misinterpreted as staining. Interpreting such a strand or other artefact as staining in Week-1 and deciding to exclude it in Week-2 is another potential cause of both inter- and intra-observer variability. Using videos for agreement experiments in the future would be expected to reduce the variability due to inconsistent interpretation of the photographs.

When image selection has been optimised, and further training has been conducted on this scale, the results from future agreement experiments may be used to further evaluate and refine the CORE corneal staining scale. Firstly, a pictorial scale would be advantageous, particularly for the *type* grade which is largely descriptive compared to the more mathematical *extent* grade. The use of pictorial references for grading scales has been recommended by several authors.[6, 7, 15] Secondly, the appropriate step size of the scale should be investigated. There is a balance to be sought between concordance and sensitivity of a grading scale; a low number of grade options provides high concordance but is not sensitive to capture small changes. Bailey *et al*.[19] reported that high concordance can sometimes indicate that the scale has too few steps and therefore is not attaining its full potential for sensitivity. It is possible that the reverse may apply to this scale i.e. there may be too many steps, which leads to poor concordance. It may be prudent to consider whether there is a need for the same granularity of step size for *type* grading along the entire scale, particularly as the inter-observer agreement worsened as the grade increased. Within a research environment, any *type* staining graded at or above 75 would almost certainly represent an adverse event and, as such, this data would be excluded from the planned research analyses. This presents a potential argument for contracting the scale such that grades of 75 and higher are instead assigned to the highest 'bucket' on this contracted scale. However, while reducing the steps in the more descriptive type scale may provide some advantages and lead to better agreement, there is significant value in maintaining the extent grade as a pseudo percentage, because it provides valuable descriptive quality in quantifying the spread of staining across the corneal surface.

Chapter 4
Geographic Distribution of Corneal Staining in Symptomatic Dry Eye

THIS CHAPTER HAS BEEN SUBMITTED TO THE OCULAR SURFACE JOURNAL.

4.1 Abstract

Purpose: To describe the geographic distribution of corneal fluorescein staining across the five corneal zones, among non contact lens wearers who report symptoms of dry eye.

Methods: Prior studies conducted at the Centre for Ocular Research & Education, Canada, were reviewed for inclusion in the analysis. Each study assessed dry eye symptoms using OSDI and also assessed corneal fluorescein staining at study entry in five zones. For each subject, the corneal zones were ranked 1-5 according to their relative staining grade, Rank-1 representing the highest grade.

Results: Data from 13 studies and 368 subjects were included in this analysis. The total number of zones assigned Rank-1 designation was 449. The inferior zone had the most Rank-1 counts of all zones (193/43%). The nasal zone had 77/21%, followed by temporal (69/16%) and superior zones (63/14%). The central zone had the lowest count of Rank-1 designations, at only 47/13%. The distribution of the observed data was tested against a model where the probability of staining arising in any zone was equal (H0), and was rejected (Multinomial LLR: $p<0.001$), therefore the higher Rank-1 count in the inferior zone was statistically significant.

Conclusion: Based on these results, in the presence of dry eye symptoms, the inferior zone typically presents the most severe grade of corneal staining. This knowledge is valuable when developing a strategy to treat dry eye signs as the inferior zone has the highest grade of staining, thus it has the potential to exhibit the greatest reduction in staining post-treatment.

4.2 Introduction

Dry eye disease is extremely prevalent worldwide with the prevalence ranging from 5 to 50%.[1] The extensive Women's Health Study reported a prevalence of almost 8% in the female US population aged 49 years and over.[2] A meta analysis conducted by the Tear Film and Ocular Surface Society (TFOS) DEWS II epidemiology subcommittee confirmed that prevalence increases with age, however signs showed a greater increase per decade than symptoms.[1]

Dry eye has been redefined by the DEWS II Report as "Dry eye is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles".[3] Although the DEWS II definition of dry eye disease precludes the necessity for corneal staining to be present, it does state that both symptoms and signs of ocular surface damage are required for a diagnosis, and that the degree of staining is an important aspect of severe dry eye disease.[3] Although not exclusively so, dry eye symptoms are frequently associated with corneal staining, which is regarded as a standard test to visualize the extent of corneal damage and assess the severity of the dry eye disease.[4, 5] The panel of the ODISSEY European Consensus Group agreed that the severity of dry eye disease can be identified from just two criteria, symptomology and corneal fluorescein staining,[6] and this combination of assessments is frequently combined in optometry and ophthalmology practice to assess dry eye.[7]

Corneal staining may be graded over the entire cornea as a whole, or in each of five corneal zones: superior, inferior, nasal, temporal, central.[8] Several publications have described the corneal staining in subjects with and without dry eye to be more common in the inferior or vertical aspect of the cornea rather than the horizontal.[9-12] Chalmers *et al.*[10] reported that the presence of > grade 1 overall as well as inferior zone staining was a useful indicator to distinguish 'normals' from those with dry eye.

To aid development, comparison and monitoring of dry eye treatments, it is valuable to understand which of the five corneal zones typically exhibit the highest grade of fluorescein corneal staining, relative to other zones. Determining the zone with the highest mean staining score in a group of subjects exposes the result to bias as it is readily influenced by the actual degree of staining. This bias can be avoided by applying a ranking score to the staining in all corneal zones, for each subject. This study was developed to investigate the corneal staining from several clinical trials previously

conducted at the Centre for Ocular Research & Education (CORE), at the University of Waterloo, in order to illustrate the distribution pattern of staining in subjects exhibiting symptoms of dry eye.

4.3 Methods

4.3.1 Selection of studies

CORE began to grade corneal staining in five zones in 1995. The grading systems used prior to this provided a single overall corneal grade. Therefore the search for relevant clinical trials to include in this study was restricted to the years from 1995 to 2015.

For a clinical trial to be determined eligible for this analysis study, it needed to meet the following pre-determined inclusion criteria:

- Received ethics approval from a University of Waterloo Research Ethics Committee
- Been conducted in accordance with the tenets of the Declaration of Helsinki
- Conducted a documented informed consent procedure with all subjects
- Recruited non-contact lens wearers
- Determined dry eye symptomology at a baseline visit using the Ocular Surface Disease Index (OSDI)
- Assessed corneal staining using sodium fluorescein viewed using cobalt blue light and a yellow barrier filter.
- Evaluated corneal staining at a baseline visit and graded in five zones; temporal, superior, nasal, inferior and central.[8]

More than 50 clinical studies were reviewed and 13 were deemed eligible.

All subject data sets of the identified trials were further reviewed to confirm their data were complete and that their symptom score indicated dry eye, regardless of the level of staining. There was no requirement of a minimum level of corneal staining. Subjects with diagnosed Sjogren's syndrome were excluded, as were all contact lens wearers.

4.3.2 Symptomology inclusion criteria

All eligible clinical trials used OSDI to assess symptomology. This questionnaire has been validated[13] and subjects are considered to have *no dry eye* if their score is 12 or less, *mild dry eye* if their score is

13 to <23, *moderate dry eye* if their score is 23 to <33, and *severe dry eye* if their score is 33-100.[14] To be included in the analysis, subjects had to have an OSDI score of 13 or more, regardless of the level of corneal staining they exhibited.

4.3.3 Corneal staining

There was no requirement of a minimum level of corneal staining for data to be included in the analysis cohort. Due to the highly dependent nature of corneal staining between eyes, only staining data from the right eye was analyzed.

Use of the same staining grading scale was not a requirement for inclusion in this analysis, only that the five corneal zones were graded separately. Three methods of reporting corneal staining were used in the identified studies, two based on a 0-100 scale and one using a 0-4 scale:

1) CORE 3-factor grading[15]: Each zone is graded for staining type (0-100, integer steps), extent (0-100, integer steps) and depth (0-4, integer steps). Each zone can be assigned a zonal stain score (ZSS) which is the product of the type, extent and depth grade (0-10,000). For all factors 0 represents no staining.
2) CORE 2-factor grading[15]: Each zone is graded for staining type (0-100, integer steps), extent (0-100, integer steps). Each zone can be assigned a zonal stain score (ZSS) which is the 2 factor product of the type and extent grade (0-10,000). For all factors 0 represents no staining.
3) 0-4, integer steps, grading: 0 = no staining or a single punctate stain; 2 = superficial micropunctate stain; 3 = macropunctate staining or minimal coalescence; 4 = coalesced staining over half the zone or more.

For each subject, the five corneal zones of the right eye were ranked from Rank-1 to Rank-5 according to the degree of staining, where Rank-1 represented the highest level of staining of that particular eye, irrespective of the actual staining grade. Where zones within a cornea were graded equally, they were assigned the same Rank-number. Therefore some eyes were awarded Rank-1 in more than one zone. Here is an example to explain this process further. If the central and inferior zones were graded equally at the highest staining grade, the nasal and temporal zones were graded equally at a lower grade and the remaining superior zone was graded at the lowest value of all, then the zones would be allocated the following ranks:

- Central & inferior: both designated as Rank-1 (highest)
- Nasal & temporal: both designated as Rank-2

- Superior: designated as Rank-3

Where a cornea did not exhibit any staining in any of the zones, no ranking was assigned and therefore these eyes are not included in the results reporting staining incidence by rank. In those corneas where all zones were graded equally, all zones were allocated Rank-1. Thus, there are more Rank-1 designations than there are subjects in the cohort.

4.3.4 Statistical analysis: Bayesian estimation of the proportion counts

A Bayesian approach was taken to analyse the data, because this paper describes the distribution of the staining and generate credible estimates for the proportion of staining observed with each pattern of staining and in each zone. A null hypothesis testing and/or confidence interval paradigm were not used for the analysis because the former tests only a single model or point estimate, and the latter does not provide a distribution of the credible values.

The observed data was taken as representative of the underlying distribution of staining across zones. The analysis determines whether pattern (ie. with or without central staining) or zone (peripheral spatial area) contribute independent influences on the probability distributions. In addition, we generated posterior predictions, with credible intervals, for the proportion of counts in each zone.

Data were included from individuals in the total sample who exhibited Rank-1 staining in only one peripheral zone of the cornea, with or without Rank-1 staining in the central zone. Therefore, for the data included in the model, each count represents a single individual and all counts were independent of each other. This sub-sample comprised 171 individuals of the total sample (n=368).

The count of the staining in each peripheral zone is the predicted variable. It was based on two predictor variables: (i) the pattern of staining, i.e. whether it includes central staining or not, and (ii) the zone of staining, i.e. where in the non-central region the staining occurred (SUP, INF, NAS, TEMP). Both predictors are nominal in scale. The data were arranged as a contingency table with 8 cells (4 zones x 2 patterns).

Specific contrasts were made between pattern of staining and zones of staining. To undertake a contrast, joint probability distributions of the credible differences were generated with 95% highest density interval (HDI). For all contrasts, a broad region of practical equivalence (ROPE), was set up around a difference of zero with margins of ±0.40 (i.e. ±50% change in the counts). If the ROPE

completely overlaps with the 95% HDI of the differences, then the contrast is practically equivalent to zero difference (analogous to 'accepting the null' hypothesis in inferential statistics). Conversely, if the ROPE does not overlap with the 95% HDI of the differences then the contrast is credibly different (analogous to 'rejecting the null'). The Bayesian analysis was carried out using modified source code from Kruschke[16] in the R statistical programming software[17].

4.4 Results

Data was pooled from 13 eligible clinical trials. Subjects were included in the analysis if their response to the OSDI survey indicated they fell into the mild, moderate or severe dry eye categories.[14] Those that were categorized as 'normal' (ie. score of 12 or less) were excluded from the analysis. The total number of eligible subjects (N) was 368.There were 106 in the mild group (OSDI scores 13-22), 98 in the moderate group (OSDI score 23-32, inclusive) and 164 in the severe dry group (OSDI score 33 and above).

104 subjects in the study sample had no staining in any of the five corneal zones. In this group the mean OSDI score was 29.8 (SD ±13.9; range 12.5 - 83.3). Comparative data for the remaining 264 subjects with staining in one or more zones demonstrated a mean OSDI of 34.8 (SD ±15.8; range 12.5 - 93.8).

4.4.1 Areas of worst staining

The number of zones ranked as Rank-1 was 449 in the total subject pool of 368. The count and proportion of Rank-1 are presented in Table 4-1 and Figure 4-1. Of all the zones, the inferior zone was most frequently graded as having the highest level of staining; the inferior zone was designated as Rank-1 a total of 193 times which represents 43% of the total count of the Rank-1 zones. The zone with the second highest frequency of Rank-1 staining was the nasal zone, which totaled 77 instances out of 449 (17%). As described above, it should be recognized that there are more zones with a Rank-1 than there are subjects (449 zones designated Rank-1 across the 368 subjects), because a single subject could have more than one zone with Rank-1 staining.

Table 4-1: Incidence by corneal zone for: Rank-1 AND zero staining in all other zones; Count of Rank-1, independent of staining in other zones; count of Rank-1 as a percentage of ALL Rank-1 counts; count of Rank-1 as a percentage of ALL subjects analyzed; mean OSDI of subjects with Rank-1 in each zone.

Zone:	Temporal	Superior	Nasal	Inferior	Central
Count of staining in ONLY one zone (zero staining in all other zones)	4	6	2	63	7
Count of Rank-1 staining, per zone	69	63	77	193	47
Count of Rank-1 staining as percentage of total number of Rank-1 zones (449)	15.5%	14.0%	17.0%	43.0%	10.5%
Percentage of subjects of total sample (N=368) with Rank-1 staining in each zone	18.8%	17.1%	20.9%	52.5%	12.8%
Mean OSDI for subjects with Rank-1 in each zone	35.0	35.1	35.3	35.4	35.9

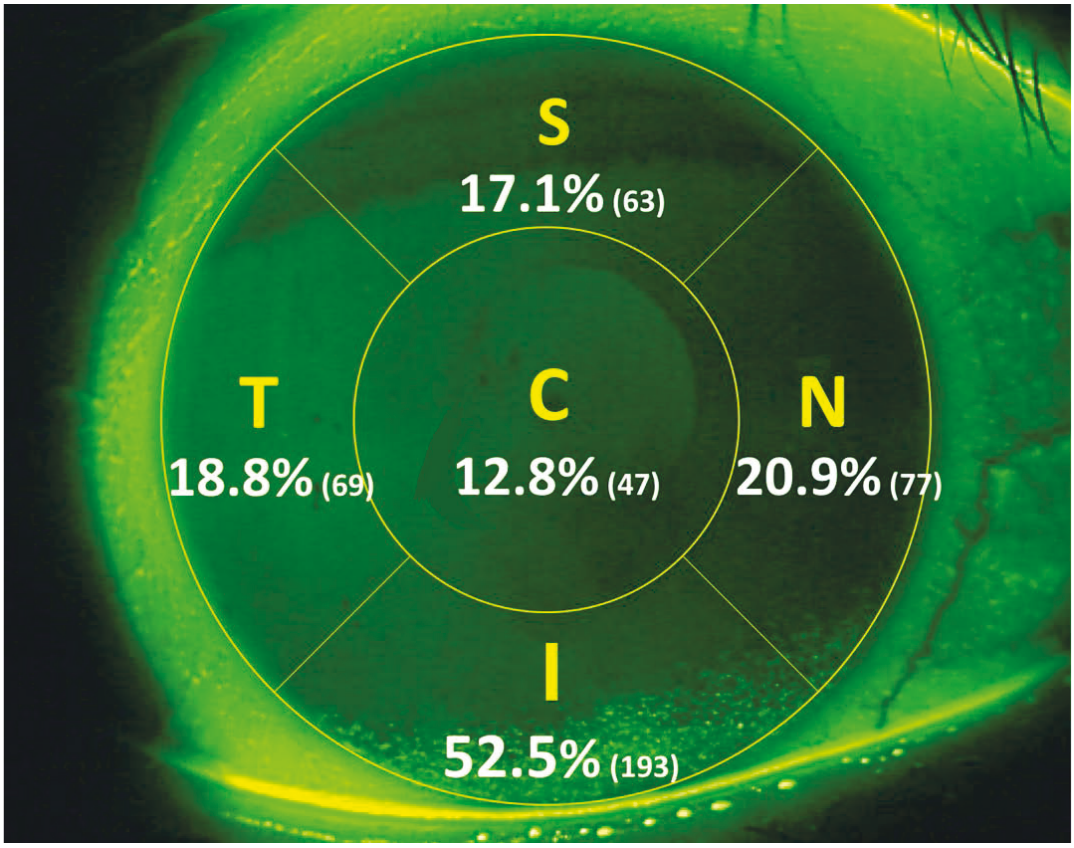


Figure 4-1: Distribution of subjects exhibiting Rank-1 (worst staining) grades in each zone, as a percentage of the total number subjects (count), n=368 subjects.

Figure 4-2 provides a graphical illustration of the distribution of Rank-1 staining (worst staining) to Rank-4, plus the distribution of zero staining, across all zones. The counts exhibiting staining of Rank-5 (lowest rank where staining is present) are not included due to the low levels of incidence. In the inferior zone, the highest count is that of the Rank-1 (worst) staining. For all except the inferior zone, the highest count is that of zero staining thus the inferior zone shows a different distribution to the other zones for Rank-1 (worst) staining.

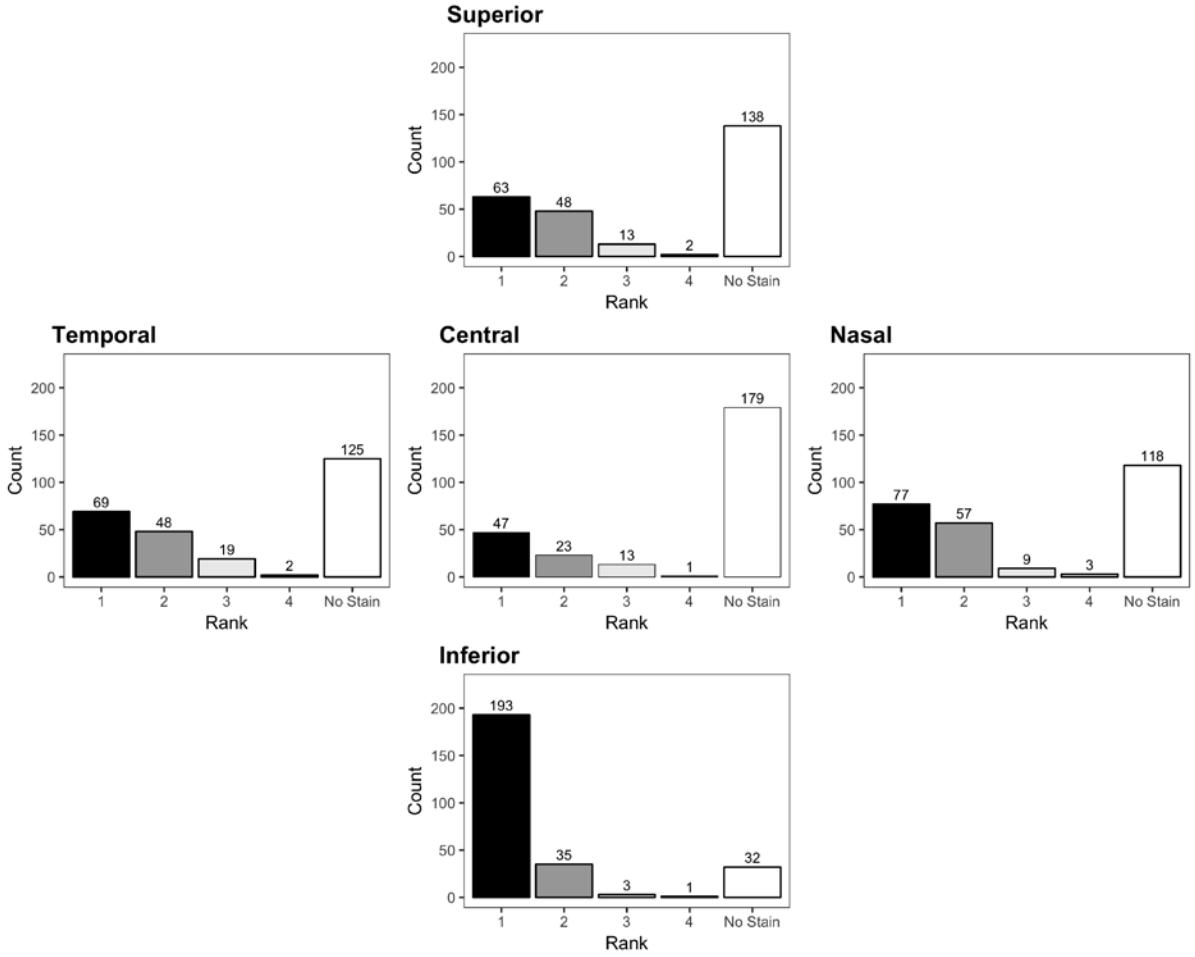


Figure 4-2: Graphical representation of counts of Rank-1 staining (worst) to Rank-4 staining plus count of zero staining, by corneal zone, n=368 subjects.

The mean OSDI score for the whole cohort was 33.4 (median 31.3; range 12.5 - 93.8). The mean OSDI for all subjects with the inferior zone staining categorized as Rank '1' (193 subjects) was 35.4. The mean OSDI was calculated for all subjects grouped by whether each zone staining was ranked '1'; the mean values were all similar, and in the lower end of the severe dry eye category, Table 4-1.

To explore the relationship between the central zone and the peripheral zones as a group as well as individually, the incidence of some specific staining patterns involving these zones were calculated, Table 4-2. This table counts all staining under the various pattern descriptions, not just Rank-1. These results clearly demonstrate that having corneal staining only in the periphery (without central zone

involvement) was far more common than the reverse observation of staining only in the central zone without peripheral zone involvement (count: 178 versus 7). Additionally, the presence of central zone staining with zero inferior zone staining only occurred in 10 subjects, whereas the reverse, i.e. staining of inferior zone staining with zero central zone staining, occurred in 157 subjects.

Table 4-2: Percentage (count) of specific staining patterns.

Staining pattern	**% subjects (N_{Total}=368)**
No staining in any zone	28.3% [104]
Staining in any zone	71.7% [264]
Staining in any peripheral zone/s only ie. no staining in central zone	48.4% [178]
Staining in central zone only ie.no staining in any peripheral zones	1.9% [7]
Staining in temporal zone only ie.no staining in any other zone	1.1% [4]
Staining in superior zone only ie.no staining in any other zone	1.6% [6]
Staining in nasal zone only ie.no staining in any other zone	0.5% [2]
Staining in inferior zone only ie.no staining in any other zone	17.1% [63]
Staining in central zone and zero staining in inferior zone	2.7% [10]
Staining in inferior zone and zero staining in central zone	42.7% [157]

It has been explained previously that, where staining was the worst and equal in more than one corneal zone, then all these zones were designated as Rank-1. Given that central zone Rank-1 incidence was so low, it was of interest to explore the patterns and zone combinations of the peripheral zone staining. The zone combinations where only two peripheral zones per eye were identified as Rank-1 (36 subjects) are shown in Figure 4-3 and the zone combinations where three peripheral zones per eye were identified as Rank-1 (25 subjects) are shown in Figure 4-4. These two graphs indicate that the inferior zone features heavily in both of these staining patterns. Twenty-one subjects had Rank-1 staining in all four peripheral zones.

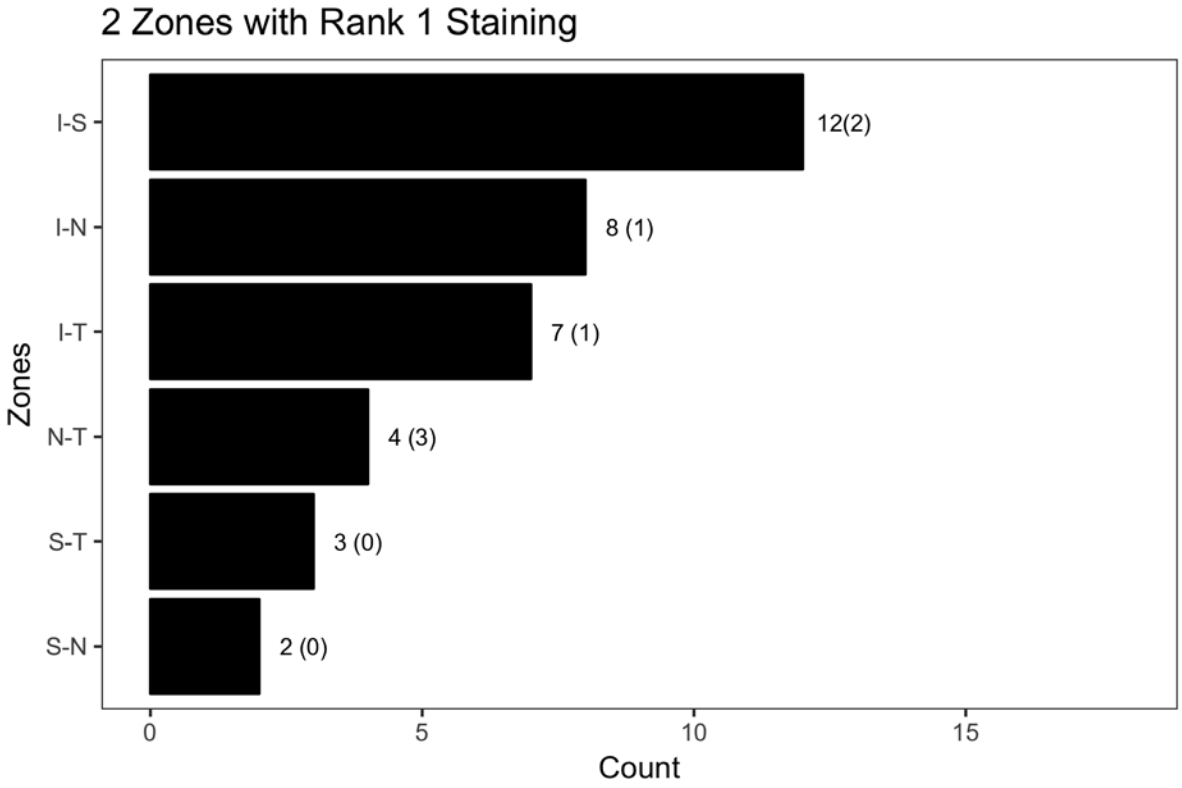


Figure 4-3: Count of eyes exhibiting two peripheral zones of Rank-1 staining, per zone combination. The number in parentheses indicates the count of eyes where each 2-peripheral zone combination presented with Rank-1 central zone staining as well.

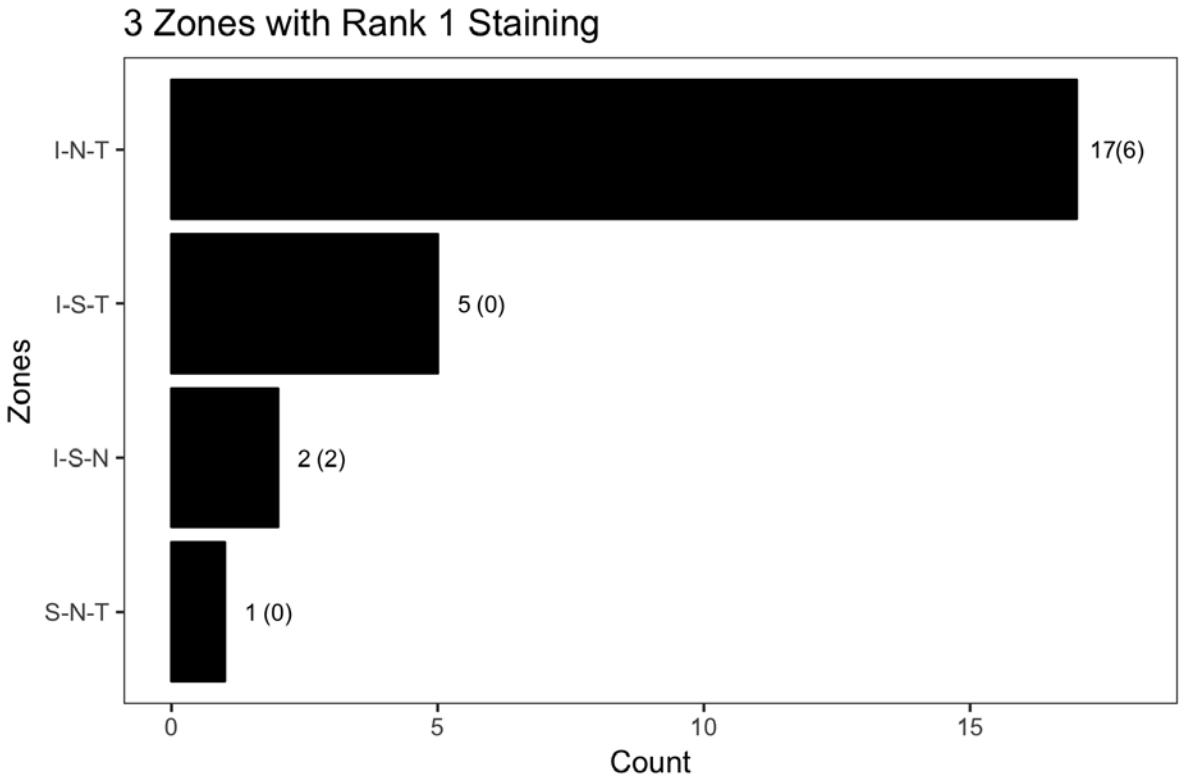


Figure 4-4: Count of eyes exhibiting three peripheral zones of Rank-1 staining, per zone combination. The number in parentheses indicates the count of eyes where each 3-peripheral zone combination presented with Rank-1 central zone staining as well.

4.4.2 Bayesian analysis

Figure 4-5 shows the posterior distributions of the count proportions for each zone within both patterns investigated: only peripheral zone staining and no central zone staining (Pattern: P); peripheral zone staining as well as central zone staining (Pattern CP). Shown on each plot is the mode of each distribution and the 95% Highest Density Interval (HDI). It can be seen that individuals presenting with a single zone of staining outside the central zone were more likely to present without central zone staining and with inferior zone staining.

Specific distributions were generated for the main effects contrasts in the model.

Peripheral staining is more likely than central staining (β deflection mode =-2.69; 95% HDI: -3.58 to -1.95, no overlap with ROPE). Considering the peripheral zones, the inferior zone is more likely to

exhibit the worst (R1) staining than the temporal (β_{IT} = 2.06; HDI: 1.07 to 3.08, no overlap with ROPE), superior (β_{IS} = 1.74; HDI: 0.81 to 2.67, no overlap with ROPE), and nasal (β_{IN} = +1.22; HDI: 0.45 to 2.00, no overlap with ROPE) zones.

For contrasts of the interactions between regions and the two patterns of staining, the ROPE and the HDI completely overlapped in all cases, indicating that the differences in staining frequency between zones was not dependent on the pattern of staining. We concluded, therefore, that the interaction term was not a major contributor to the model.

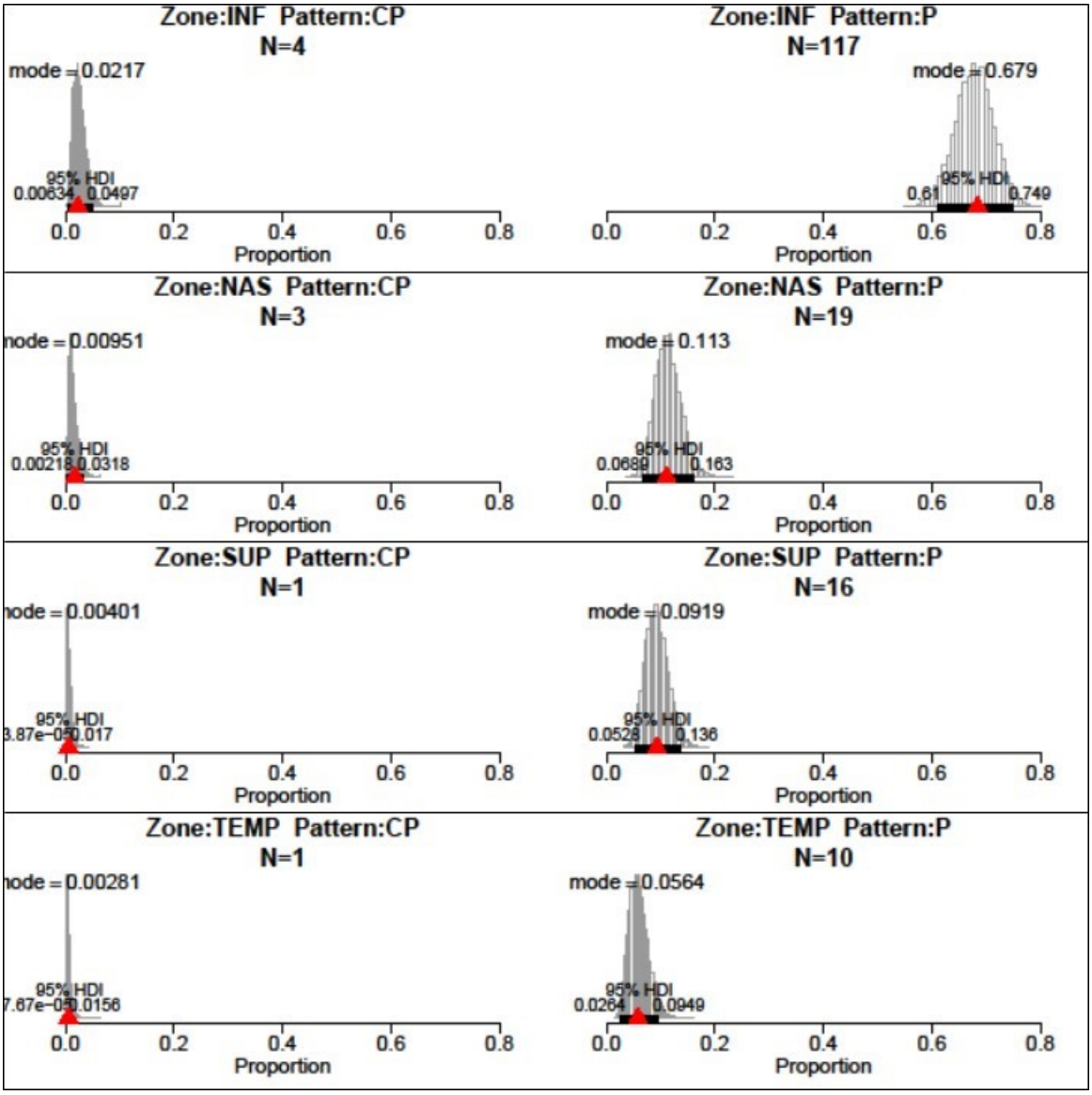


Figure 4-5: Posterior distribution of count proportions for each cell of the contingency table. Pattern: CP indicates subjects with central zone staining WITH one region of non-central zone staining. Pattern: P indicates subjects with one zone of non-central staining alone. The red triangle indicates the actual data proportions. INF: inferior zone, NAS: nasal zone, SUP: superior zone, TEMP: temporal zone.

4.5 Discussion

This study documented the frequency and pattern of staining in each of five geographic zones of the cornea (central, superior, nasal, inferior & temporal) in subjects with symptoms of dry eye disease, by retrospective review of data from 13 clinical studies conducted at CORE. Inferior zone staining was demonstrated to be the most common location for the worst staining of the entire cornea, whether the staining occurred only in one zone or whether it affected multiple corneal zones.

The findings of this study support previous evidence in the literature of inferior staining being highly indicative of dry eye.[9,11,18] Chalmers *et al.*[9] concluded that overall and inferior zone staining was useful to distinguish dry eye subjects from 'normals'. Tong *et al*[11] recently published a study assessing the effect of punctal plugs and the cohort of 29 moderately severe symptomatic dry eye subjects exhibited the highest grade of baseline corneal staining in the inferior zone. Interestingly, they also noted that the inferior zone corneal staining appeared to be more resistant to showing improvement with the study treatment of punctal plugs. While this current study does not report severity of staining, the highest frequency of worst staining being in the inferior zone aligns with Tong *et al*'s results. Tong *et al* reported the least severe staining in the superior zone and while this current study found the lowest Rank-1 incidence in the central zone (demonstrated in 12.8% subjects), the superior zone was the second lowest, demonstrating Rank-1 in 17.1% subjects.

There are several reasons why the inferior corneal zone might show the most severe and/or the most frequent staining of all the corneal zones. Firstly, the inferior zone is the one zone that is left exposed in situations of incomplete blinking. Incomplete blinks approximately double the duration of exposure to evaporation for the over-exposed inferior ocular surface.[19] Secondly, while the other zones undergo a full wiping effect from the upper lid during the blink movement, which may help to remove toxins from the corneal surface and helps to re-establish the tear film, the wiping of the inferior zone is more limited because the inferior lid remains fairly static with virtually no wiping effect.[20] Because of this limited movement of the lower lid, more tear film debris and bacterial toxins from the lid may collect along the lower lid margin, especially in those dry eye subjects with reduced a tear volume that can flush these away.[21] There are conflicting reports about whether the ocular bacterial load is higher in subjects with dry eye compared to those without dry eye, with variability in the results likely due to variations in the bacteria studied, and also with the confounding presence of blepharitis and meibomian gland disorders.[22-24]

The methodology used in this study ranked the staining per zone of each cornea, thereby removing the impact of differing grading systems being used across the studies. Additionally, all the scales provided central versus peripheral staining data. By these means, estimates of the proportions of the location at which the worst staining presented were able to be generated for dry eye subjects with symptoms (Figure 4-1). It is clear from the data that central staining is not the most common presentation, but this does not imply that central staining is unimportant given its proximity to the visual axis. Nevertheless, its low frequency does not make it a good metric to evaluate the performance of treatments to alleviate staining in dry eye. This illustrates that it is critical to choose a metric that has a scalar extent appropriate for the response that is being evaluated.

This study provides further support for the inferior zone being the zone most commonly affected by corneal staining in people suffering with dry eye. As the most commonly affected zone it becomes imperative to evaluate this zone for monitoring treatment effects which has not always been the case in previous dry eye research. In order for a dry eye therapy to become approved, the U.S Food and Drug Administration (FDA) specify that an improvement should be proven in both symptoms and one other validated sign.[25] The method of capturing symptomology is not specified. The validated sign is not restricted to corneal staining and could be other signs related to the dry eye condition, for example Schirmer test, conjunctival staining or bulbar conjunctival redness among others, though corneal staining has commonly been used.

In the past 15 years only two therapeutic topical dry eye treatments have been successful in their application for this indication from the FDA; cyclosporine in 2003 (Restasis, Allergan) and lifitegrast in 2016 (Xiidra, Shire). In the 13 years between these products coming to market, more than a dozen compounds aimed at treating dry eye have initiated the FDA process for approval as a dry eye drug but have failed to show efficacy.[26] The lack of correlation between dry eye signs and symptoms[27] has meant that demonstrating improvement in both factors in just one study has been challenging. Some dry eye patients could be highly symptomatic but show fewer signs of the disease, while other patients may show significant signs, such as corneal staining, but are less symptomatic. Results from this study provide further evidence that corneal staining and symptoms do not always co-exist. All subjects in this study were symptomatic of dry eye however 104 (28.3%) of the 368 subjects did not show any corneal staining at all.

Restasis, had its treatment efficacy proven based on symptoms, which included using OSDI, plus Schirmer test as the sign.[28, 29] Notably, a review of clinical evidence from the early randomized

controlled trials showed that Restasis also decreased corneal staining in the majority of these trials.[28, 29] The more recent FDA approval of Xiidra was very interesting because it was the first approval where the improvement in the dry eye symptom and the dry eye sign were accepted despite them each being demonstrated in separate studies. The Xiidra application was supported by symptoms collected via visual analogue scales (burning/stinging, itching, foreign body sensation, eye discomfort, photophobia, pain) and an overall ocular discomfort score.[18, 30, 31] The clinical sign demonstrating effectiveness was the inferior corneal staining score.[30]

4.6 Conclusion

In conclusion, this study demonstrates that the inferior zone most commonly exhibits the most severe staining in symptomatic dry eye disease. Given this information, although severe central zone staining is potentially more important for visual outcomes, studies investigating dry eye disease treatments should consider targeting improvements in corneal staining in the inferior zone specifically, in order to be more impactful across the spectrum of sufferers likely to be included in clinical trials.

Chapter 5
Effect of Lens and Eye Rinsing on Solution Induced Corneal Staining (SICS), a Pilot Study

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5.1 Abstract

PURPOSE: The main purpose of this study was to determine whether two interventions (rinsing the lens prior to lens insertion and rinsing the ocular surface post lens removal) had any impact on solution induced corneal staining (SICS). In addition, the presence of hyper-reflective epithelial cells in the presence of SICS was investigated.

METHODS: Twenty subjects wore new balafilcon A lenses, which had been soaked overnight in a multipurpose care product containing polyhexamethylene biguanide for 2-hours. The study was conducted across three phases. In Phase-1 (investigator and subject masked, randomized eye), one lens was rinsed with non-preserved saline prior to lens insertion. In Phase-2 (investigator masked, randomized eye) one eye was rinsed with non-preserved saline after lens removal, prior to staining assessment. Corneal staining was recorded as the percentage area of the cornea exhibiting superficial punctate staining. In both phases, ocular comfort and presence of specific symptoms were captured. In Phase-3, there was no randomized treatment; confocal images of the epithelium were obtained after 2-hours wear.

RESULTS: In Phase-1 (lens-rinse), there was no significant difference in staining between the treated and untreated eyes (84% vs 92%, respectively; p=0.06). In Phase-2 (eye-rinse), there was also no significant difference between the treated and untreated eye (86% vs 86%, p=0.92). Most subjects

were asymptomatic. In Phase 3, images of hyper-reflective cells were captured in 97% of the eyes imaged.

CONCLUSIONS: The two rinsing procedures did not affect the level of the SICS response. Hyper-reflective epithelial cells were found to be present in a significant number of eyes exhibiting SICS and their presence warrants further investigation.

5.2 Introduction

The development of excessive superficial punctate staining associated with the use of silicone hydrogel (SiHy) lenses and certain care regimens was initially reported in 2002,[1, 2] and has been the topic of much investigation since. The term most commonly adopted to describe this phenomenon is "solution induced corneal staining" or SICS.[3]

The initial reports on SICS described the corneal staining response when one SiHy contact lens material (balafilcon A) was worn following disinfection with a multipurpose solution which contained polyhexamethylene biguanide (PHMB).[1, 2] A subsequent clinical trial by Garofalo *et al.*[4] established that the corneal staining response was maximal approximately two hours after lens insertion.[4] Several studies indicated that the SICS response was greatest when certain SiHy and FDA group II (high water content, neutral charge) hydrogel materials were exposed to PHMB-preserved multipurpose solutions (PHMB-MPS).[4-7]

Following these initial studies, Carnt *et al.*,[3, 8] and Andrasko and Ryen[9] conducted experiments investigating the staining response to combinations of the most commonly prescribed lenses and care products. They found that some combinations led to varying degrees of SICS, while others did not. They presented their results in a grid format that indicated the varying severity of the SICS response with different product combinations.[8, 9] The work by Andrasko and Ryen[9, 10] indicated there was a greater degree of corneal involvement associated with PHMB-MPS. However, products containing the same concentration of PHMB gave rise to widely varying levels of corneal involvement,[9-12] which remains difficult to explain. It was also shown that certain lens materials in combination with PHMB-MPS led to higher amounts of SICS than other materials.[9, 10, 12] These grids highlighted the fact that SICS was not associated with specific lens materials or products, rather it was associated with specific combinations of lens materials and care products.

The symptoms associated with SICS have been reported as being both non-existent and significant. Though the initial publications referred to SICS as an asymptomatic phenomena,[1, 2] the Jones *et al.*[2] paper did report stinging symptoms on lens insertion. SICS has more recently been described as being associated with the whole spectrum of symptoms from none,[13] to both mild[4, 9] and more significant symptoms.[14, 15]

Retrospective analyses of over 600 participants in clinical trials have shown that SICS is associated with a higher frequency of infiltrative events.[16-18] A more recent prospective clinical trial of 19 participants with induced SICS revealed that inflammatory markers in the tears were up-regulated, suggesting that there may be an active ocular surface inflammatory response.[19] If SICS does indeed lead to an inflammatory response, rather than simply being associated with it, then its presence would raise concern. However, the co-existence of infiltrates and SICS could be coincidental rather than SICS being causative of infiltrates, and recent work has not been able to link SICS and tear film inflammatory changes.[20] This uncertainty of a link with adverse responses fuels continued investigation of SICS.

Recently, Bright *et al.*[21] have offered a hypothesis which has become known as PATH; "preservative associated transient hyperfluorescence". This hypothesis explains the appearance of solution-related corneal staining as being caused by the release of PHMB molecules out of the contact lens onto the ocular surface,[22, 23] which then bind to the corneal epithelial cells, with fluorescein then binding to these epithelial-bound PHMB molecules. The hypothesis further suggests that this fluorescein-PHMB-epithelium complex is inert and does not create any physiological distress at the cellular level. The reports of minimal associated symptoms seem to support an inert process taking place. However, Bright *et al.*'s theory[21] was developed using an in-vitro liposomal-based model and has not been replicated in the human eye.

The PATH theory[21] is somewhat compelling[24] and may help to explain some of the aspects of SICS, notably the lack of major symptoms and the kinetics of the staining response, which demonstrate a peak at two hours and subsequent gradual reduction over time.[4] However, a few unexplained findings remain. If a generalised fluorescein-binding to surface-bound PHMB was occurring, then it would be expected that the staining appearance would be fairly diffuse and evenly distributed over the entire cornea. However, it is often seen in a "doughnut-like" annulus,[2, 12, 14] with minimal staining centrally and more exaggerated staining peripherally. The PATH theory only holds when fluorescein is present. However, when SICS is induced, changes can be observed on the corneal

surface under white light conditions, when no fluorescein has been instilled. These white/grey punctate disturbances of the cornea appear to mimic the areas highlighted with subsequent fluorescein instillation, as described by Bandamwar and colleagues[25] and Maldonado-Codina *et al*.[26] In addition, so called "hyper-reflective cells" determined by clinical confocal microscopy have been shown to be associated with SICS.[27-31] These cells can be observed without fluorescein[29, 30] and their level of reflectivity suggests that the cells are dying.[31] These two epithelial cellular observations, that are both observed without fluorescein, provide contrary evidence to the PATH theory, which suggests that there is nothing untoward happening to the superficial epithelial cells.

Bright *et al*.[21] suggested that fluorescein innocuously binds to the outer cell membrane. However, following micro-biopsy of a fluorescein-staining cornea, fluorescein has been imaged within the epithelial cell cytoplasm, confirming physiological changes within the cell.[32] Additionally, fluorescein-stained cells collected non-invasively from a cornea exhibiting SICS can be imaged by a laboratory scanning confocal microscope. This method has demonstrated that fluorescein is present throughout the cell, and was not merely bound to the outer membrane.[33]

Clinical observations such as those detailed above, along with the ensuing clinical and laboratory trials, have caused the research community to reconsider what corneal staining with fluorescein actually represents.[24, 34-36] Whereas it was commonly believed that fluorescein was highlighting dead epithelial cells, it now appears that cells must be alive with active metabolism in order to absorb fluorescein and become 'stained'.[24, 31, 32, 36, 37] One study examined the fluorescein stained cells washed from corneas exhibiting SICS and found them to be in a cycle of pre-programmed cell death, called apoptosis.[38] It is as yet unclear whether these "stained" cells are entering apoptosis at a faster rate than they normally would. Despite uncertainties about the mechanism behind SICS and its symptomatology, the major question of whether it is clinically relevant remains elusive.

The purpose of this study was to determine whether two specific interventions had any impact on the level of SICS. The first intervention was a thorough rinsing of the lens before it was inserted onto the ocular surface and the second intervention was a thorough rinsing of the eye following lens removal and prior to fluorescein insertion. The hypothesis was that both of the rinsing interventions would remove a significant portion of any PHMB adhered to the lens (prior to wear) or the corneal epithelium (post-wear), such that the level of SICS would be measurably reduced in the treated eye compared to the untreated eye. If the hypotheses were true, a recommendation to patients to rinse their lens or eye would be beneficial in reducing the SICS response. The study design also offered an

opportunity to investigate the presence of hyper-reflective (HR) epithelial cells alongside SICS, using in-vivo confocal microscopy.

5.3 Methods and materials

5.3.1 Participants

Twenty experienced soft contact lens wearers aged 17 years or older with good general and ocular health were recruited. All participants were required to demonstrate a good fit with the study lens at a screening/eligibility visit. Informed consent was obtained from all participants prior to enrolment in the study. The trial was conducted in accordance with the Declaration of Helsinki and ethics clearance was obtained through the Office of Research Ethics at the University of Waterloo, prior to commencement of the study.

5.3.2 Study outline

This study was a prospective, non-dispensing design with three phases. The order of the phases was not randomised. In phases 1 and 2, the eye assigned to the treatment was randomly assigned and also masked to the primary investigator. Each phase was preceded by a minimum of 24-hours of no contact lens wear and the phases were separated by a minimum of one week, during which habitual contact lens wear was allowed. During each phase, participants bilaterally wore a pair of new balafilcon A lenses (PureVision™; Bausch & Lomb, Rochester, NY) which had been pre-soaked overnight (for approximately 15 hours) in a 0.0001% PHMB-preserved MPS (renu® fresh™; Bausch & Lomb, Rochester, NY). To aid consistency of exposure across participants irrespective of their prescription level, all lenses worn in the study were of power -0.25D, and participants wore their spectacles over the top. Prior to beginning each phase, the corneas were assessed for epithelial disruption under white light, without instillation of sodium fluorescein. This assessment was conducted to ensure the cornea was healthy and able to wear a contact lens. Instilling sodium fluorescein was avoided prior to lens wear in order that there was no fluorescein present in the eye to potentially react with the PHMB following lens insertion.

In Phase 1, one lens was inserted directly from renu® fresh™ (untreated) and the other lens was thoroughly rinsed in a non-preserved, borate-buffered saline (Unisol 4; Alcon, Fort Worth, Tx) prior

to lens insertion (treated). A new bottle of Unisol 4 was opened on each study day. The treatment lens was held in tweezers and rinsed on both sides under a stream of saline for at least five seconds, then dipped into a well of fresh saline before being picked up by the tweezers on a new region of the lens and being rinsed on both sides again under a second stream of saline for at least another five seconds. The treatment lens was not rubbed. Both lenses were worn for two hours before being removed for corneal fluorescein staining grading and ocular assessment. The eye that wore the treated lens was assigned according to a randomisation table and this was masked from both the participant and investigator by enlisting a second investigator to conduct the lens rinsing.

In Phase 2, both lenses were inserted directly from renu® fresh™. After two hours of lens wear, the lenses were removed and one eye (untreated) received no rinsing prior to fluorescein staining assessment, while the other eye (treated) was thoroughly rinsed for 3-5 seconds on three successive occasions with Unisol 4 prior to fluorescein staining assessment. Once again, the treated eye was assigned according to a randomisation table and this was masked from the primary investigator by enlisting a second investigator to conduct the eye rinsing. Following rinsing, the primary investigator conducted the grading of the corneal staining and ocular assessment.

In Phase 3, no lenses or eyes were rinsed and both eyes wore lenses that had been pre-soaked overnight. This phase investigated the presence of HR superficial epithelial cells and was observational in nature, with no direct control for comparison. The pre-soaked lenses were worn for two-hours and the anterior epithelial cell layer was imaged through the contact lens using a confocal microscope (Confoscan 3; Nidek, Japan) without instillation of anaesthetic. Anaesthetic was initially avoided to eliminate any potential image artefacts or epithelial disruption, which may have been caused by either the anaesthetic or associated preservatives. Following lens removal, the participant's cornea was then anaesthetised with two drops of a topical anaesthetic (Alcaine 0.5%; Alcon Canada Inc, Mississauga, Canada) and the epithelium was re-imaged using the confocal microscope. Following this second confocal imaging session, corneal fluorescein staining was graded.

5.3.3 Assessment technique for corneal epithelial disruption and staining

Corneal staining was assessed on four occasions, three without and one with fluorescein, during each phase. At the beginning of each phase, prior to lens insertion, the cornea was observed with a biomicroscope using a broad beam under white light, at 12x magnification. Any areas of reduced transparency were recorded as being white light observations of epithelial disruption, or "white light

staining". Corneal disruption was assessed under white light again after two hours of lens wear, first with the lens in situ and then again after lens removal. The fourth observation was made following the instillation of sodium fluorescein via a fluoret, under 12x magnification, through a yellow barrier filter using cobalt blue illumination.[39]

During the pre-lens "white light staining" assessment, no grading was attempted but the mere presence or absence was noted, and images were taken. For the fluorescein staining assessment, the area of the cornea exhibiting disruption or staining was graded as a percentage, in each of five zones; temporal, nasal, superior, inferior and central.[40] The percentage areas in these zones were then averaged to provide the area of disruption across the entire cornea. This subjective area assessment is a standard method of corneal staining assessment at CORE,[41] and all assessments were conducted by a single investigator with over seven years of experience with this methodology.

5.3.4 Assessment technique for limbal hyperaemia

Limbal hyperaemia was graded under diffuse white illumination and 8x magnification. The level of hyperaemia was graded separately for each quadrant (temporal, nasal, inferior, superior) according to the CORE 0-100 integer grading scale, where 0 indicates completely white tissue. This subjective hyperaemia assessment is the standard method of hyperaemia assessment at CORE, and all assessments were conducted by a single investigator with over seven years of experience with this methodology. Assessments took place twice during each phase, first before lens insertion and secondly immediately before the grading of corneal staining, which was after the eye rinsing step in Phase 2.

5.3.5 Symptomatology

During phases 1 and 2, participants were asked to report, for each eye, an overall symptom score (using a 0-100 integer scale where 0 represented 'very uncomfortable' and 100 represented 'very comfortable'). They were also asked whether three specific symptoms were present or absent; burning, stinging and itching. Participants were asked about their symptomatology on four separate occasions during each phase; immediately before and after lens insertion, and immediately before and after lens removal.

5.3.6 Assessment technique for hyper-reflective (HR) cells

The Confoscan 3 was set to take an epithelial scan using the automatic focus mode. An attempt was made to collect at least one full scan of the central cornea per participant, both with the contact lens in situ (without anaesthesia) and then following lens removal, after instilling two drops of Alcaine anaesthetic. Although the central cornea was targeted in each case, the exact location on the cornea could not be exactly specified. Thus the locations that were sampled with and without the contact lens in situ may have differed slightly. The microscope captures a set of 350 images per complete scan, with each image offering a viewing area of 450 x 340 μm at a magnification of 500x. Each image within these scans was viewed individually to determine whether it was positive for HR epithelial cells. For each positive image, the number of HR cells was recorded. Because the number of positive images could vary from zero to over thirty in an individual scan, the data presented in this manuscript is the maximum count of HR cells viewed in any one image, for each participant.

5.3.7 Data analysis

All data was analyzed by CORE at the University of Waterloo using Statistica 10. Descriptive statistics are given for the participants' age and gender, and also for the presence of specific symptoms and the count of HR cells. Analysis using paired t-tests were applied to compare corneal staining areas and symptom ratings. Changes in symptom ratings over time were analysed using repeated measured ANOVA, with Tukey as the post-hoc test. An alpha value of 0.05 was considered to indicate a statistically significant difference.

5.4 Results

All twenty participants completed the study; all were female, mean age of 34.2 years (median 34.5 years, ranging from 18 to 64 years).

5.4.1 Corneal staining

During Phases 1 and 2, before lens insertion, corneal assessments were made with white light, as previously described. Of the 80 baseline staining observations, most showed no white light staining at all (57), while 23 exhibited clinically insignificant white light staining (total corneal area staining ≤6%). After two hours of lens wear, initial observations of the corneas were made under white light with lenses in situ. After lens removal, white light observations were repeated. While it was possible to observe epithelial changes through the contact lens, grading and photographic capture proved challenging and no reports are included of this. Images were taken with white light following lens removal, which revealed epithelial disruption, even though no fluorescein had been instilled. All subjects showed some degree of observable white light staining in both eyes. A representative image is shown in Figure 5-1.

After images were taken under white light, corneal staining was graded under cobalt blue light, using the procedure previously described in Section 3.3. Figure 5-2 shows a representative image of the corneal staining observed. All staining presented as diffuse, superficial and punctate, covering at least four of the five corneal zones, and therefore was comparable with that expected with SICS.[1-3] Because all staining was superficial and punctate, for ease of reporting and analysis, the area of the cornea exhibiting the staining is the variable used for reporting the amount of staining. Table 5-1 and Figure 5-3 report the results of the corneal staining area for Phases 1 and 2. For each phase, after two hours of lens wear the area of staining in the treated eyes was not statistically different to that of the untreated eyes ($p=0.06$ in Phase 1, treated 84.0% and untreated 91.8%; $p=0.92$ in Phase 2, treated 86.2% and untreated 85.8%.).There was minimal staining observed under white light before lenses were inserted in each phase and as a result each phase gave rise to clinically significant increases in corneal staining following the two hours of lens wear (both $p<0.01$).

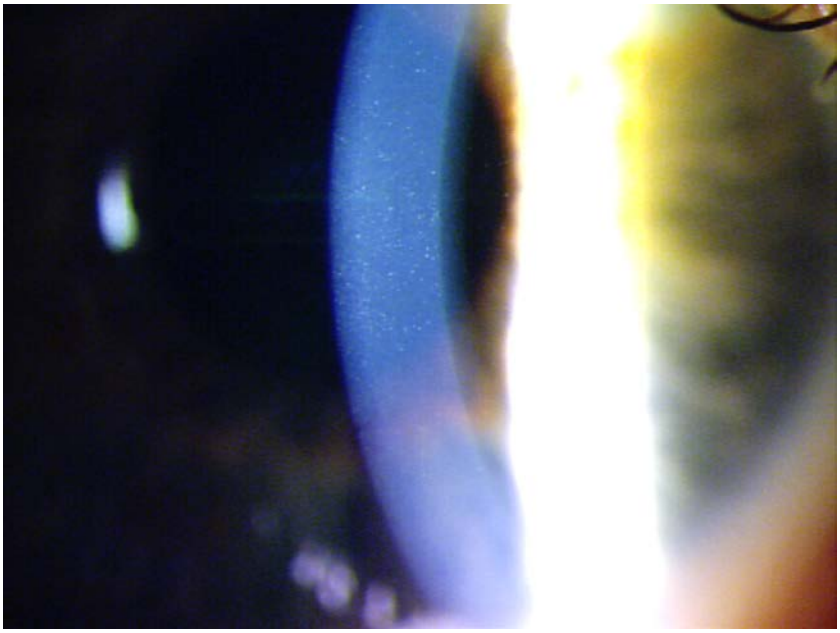


Figure 5-1: Corneal epithelial disruption ("white light staining") following lens removal and before instillation of fluorescein, after two hours of lens wear.

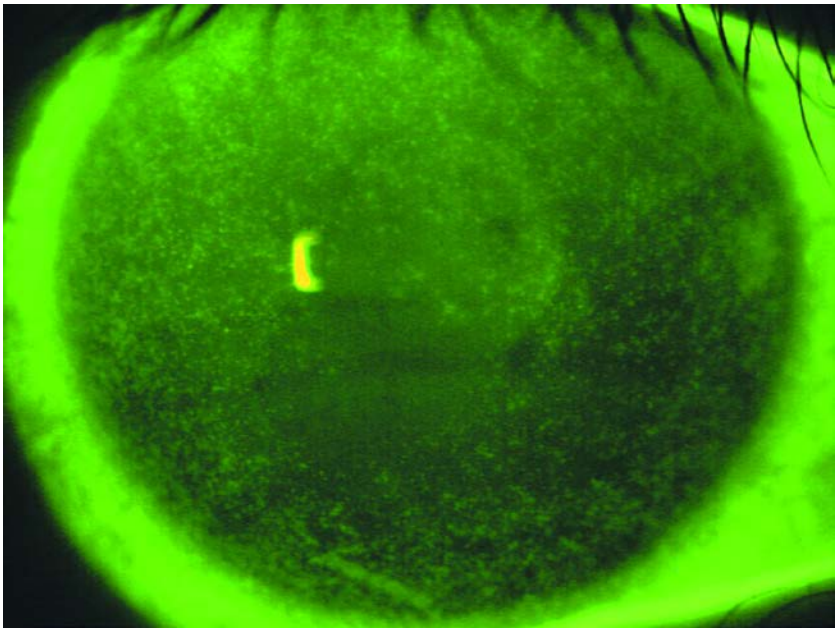


Figure 5-2: Typical example of a cornea demonstrating fluorescein staining over 85% of cornea following lens removal and instillation of fluorescein, after two hours of lens wear.

After images were taken under white light, corneal staining was graded under cobalt blue light, using the procedure previously described. Figure 5-2 shows a representative image of the corneal staining observed. All staining presented as diffuse, superficial and punctate, covering at least four of the five corneal zones, and therefore was comparable with that expected with SICS.[1-3] Because all staining was superficial and punctate, for ease of reporting and analysis, the area of the cornea exhibiting the staining is the variable used for reporting the amount of staining. Table 5-1 and Figure 5-3 report the results of the corneal staining area for Phases 1 and 2. For each phase, after two hours of lens wear the area of staining in the treated eyes was not statistically different to that of the untreated eyes ($p=0.06$ in Phase 1, treated 84.0% and untreated 91.8%; $p=0.92$ in Phase 2, treated 86.2% and untreated 85.8%.).There was minimal staining observed under white light before lenses were inserted in each phase and as a result each phase gave rise to clinically significant increases in corneal staining following the two hours of lens wear (both $p<0.01$).

Table 5-1: Percentage area of cornea (per zone: nasal, temporal, central, superior, inferior), demonstrating fluorescein staining at post-lens wear assessments (mean and standard deviation). Phase 1 treatment = one lens rinsed before wear; Phase 2 treatment = one eye rinsed following lens removal.

		Untreated eye						Treated eye					
		Nasal	Temp	Cent	Sup	Inf	Mean	Nasal	Temp	Cent	Sup	Inf	Mean
PHASE 1	Mean	94.3	93.0	93.5	88.5	89.8	91.8	90.3	87.8	77.7	78.5	86.0	84.0
	SD	8.3	12.5	17.9	14.1	11.6	8.6	10.8	16.5	35.2	24.9	16.2	17.3
PHASE 2	Mean	88.5	89.8	79.3	85.6	86.0	85.8	88.8	90.0	80.3	83.5	88.5	86.2
	SD	21.3	13.5	29.6	21.6	16.5	17.0	13.9	13.6	26.2	21.7	9.5	13.6

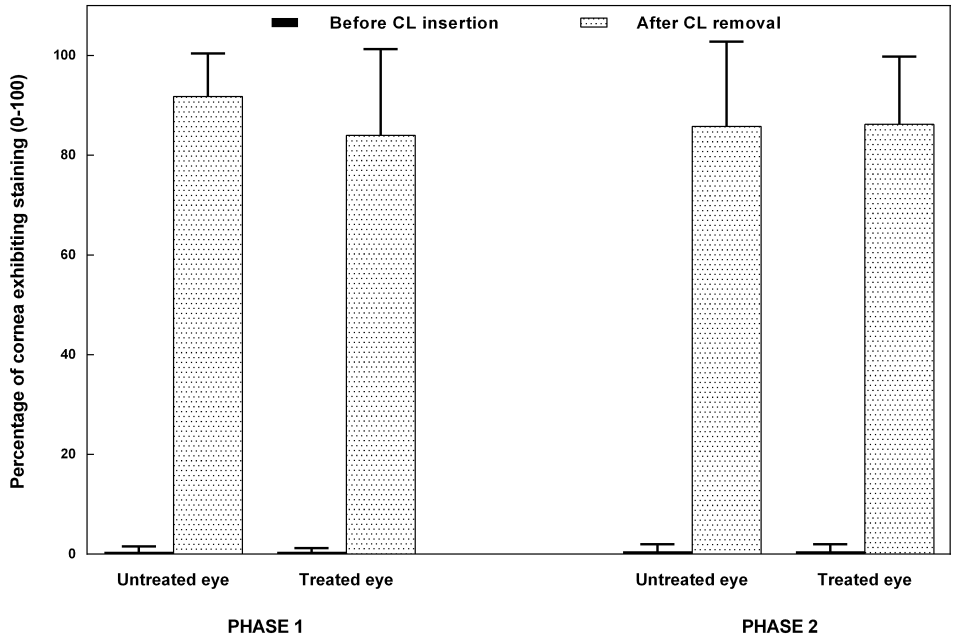


Figure 5-3: Mean & SD of the percentage area of corneal staining, before and after lens wear for the untreated and treated eyes, Phases 1 and 2. Phase 1 treatment = one lens rinsed before wear; no statistical difference between eyes after lens removal, p=0.06; Phase 2 treatment = one eye rinsed following lens removal; no statistical difference between eyes after lens removal, p=0.92.

5.4.2 Limbal hyperaemia

The mean levels of limbal hyperaemia across all quadrants, before and after lens wear, during Phases 1 and 2 are shown in Figure 5-4. For each phase, the mean level of hyperemia was within normal limits and there was no difference between treated and untreated eyes after the two-hour wear period (p=0.74 Phase 1, p=0.11 Phase 2).

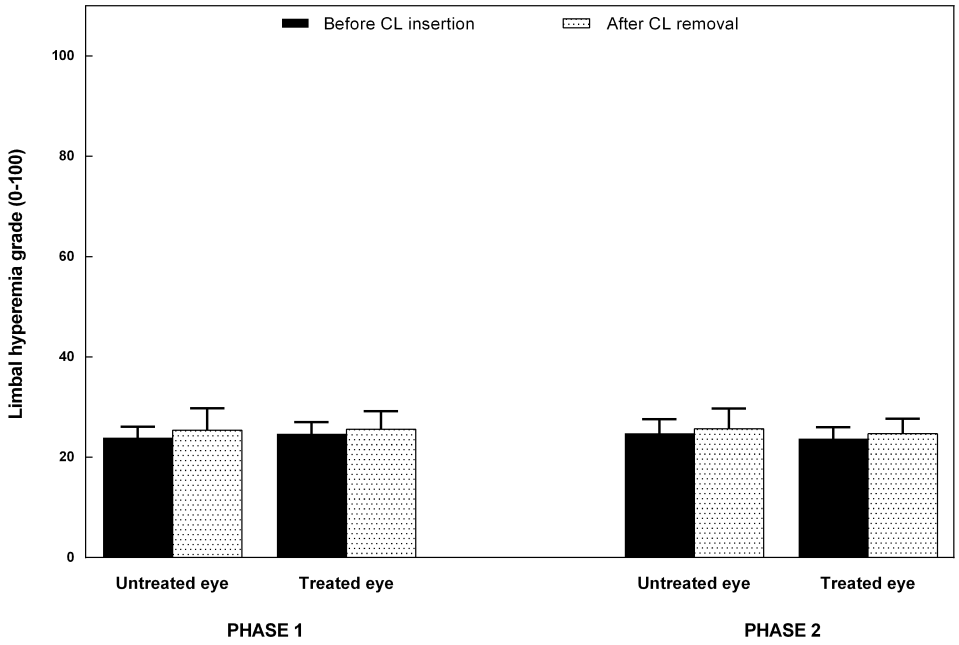


Figure 5-4: Mean & SD of the overall ocular hyperaemia grades, 0-100 integer scale where 0 is totally white, before and after lens wear for the untreated and treated eyes, Phases 1 and 2. Phase 1 treatment = one lens rinsed before wear; no statistical difference between eyes after lens removal, p=0.74; Phase 2 treatment = one eye rinsed following lens removal; no statistical difference between eyes after lens removal, p=0.11.

5.4.3 Symptomology

The numbers of participants who reported the presence or absence of stinging, burning or itching at the various time-points during Phase 1 and Phase 2 is shown in Table 5-2. The majority of participants reported no symptoms at each time-point.

Table 5-2: Count of participants during Phase 1 and Phase 2 who reported either no symptoms or symptoms of stinging, burning and/or itching at each time-point, in both the untreated and the treated eye. Phase 1 treatment = one lens rinsed before wear; Phase 2 treatment = one eye rinsed following lens removal.

PHASE	TIME-POINT	STINGING		BURNING		ITCHING		NONE	
		Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
1	Before CL insertion	0	0	1	1	1	1	18	18
	Post CL insertion	3	0	1	0	3	1	14	19
	Before CL removal	2	1	1	2	3	3	15	14
	Post CL removal	9	5	5	4	0	0	7	12
2	Before CL insertion	0	0	1	1	0	0	18	19
	Post CL insertion	2	1	0	2	1	0	17	18
	Before CL removal	1	3	3	3	2	1	14	14
	Post CL removal	2	5	3	4	1	0	15	13

The subjective ocular comfort grades (0-100) at all four time-points, for each phase, are shown in Figure 5-5. Over the two-hour wear period, in both phases, both the treated and the untreated groups showed a reduction in mean comfort score with the drop in comfort from pre-lens insertion to post lens removal as statistically significant (all $p<0.04$, ANOVA, Tukey post-hoc). There was no difference, at any time-point, in either phase, between the comfort scores of the treated and untreated eyes (all $p>0.05$).

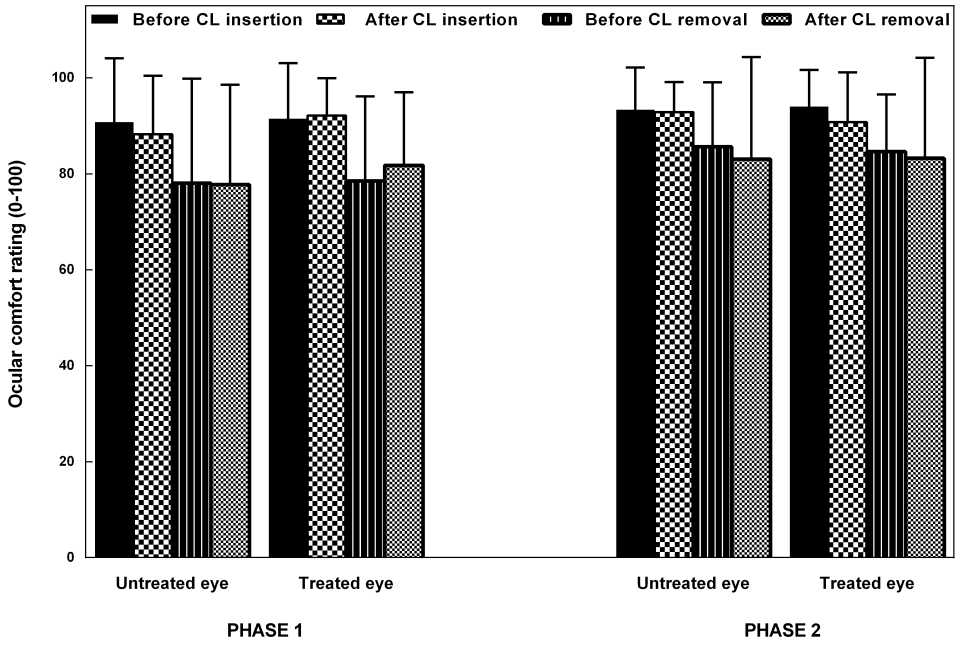


Figure 5-5: Mean & SD subjective comfort score at each time-point, 0-100 integer scale where 100 is perfect comfort, Phases 1 and 2. Phase 1 treatment = one lens rinsed before wear; no statistical difference between eyes at any time-point, all p>0.05; Phase 2 treatment = one eye rinsed following lens removal, no statistical difference between eyes at any time-point, all p>0.05.

5.4.4 Hyper-reflective cells

It was possible to image HR cells through the contact lens without using anaesthetic (Figure 5-6, left) but the images were somewhat degraded by reflections from the lens compared to the images taken following contact lens removal and instillation of anaesthetic (Figure 5-6, right). Table 5-3 shows the maximum count of HR cells in any one image for each individual. Unfortunately, due to the nature of this procedure, some participants were unable to maintain steady gaze for long enough to attain any images; in these six eyes 'n/a' was entered into Table 5-3. The entry of '0' in this table, for ID18, indicates that clear images were captured but no HR cells were observed. Of the 34 eyes that were successfully imaged, 33 (97%) were observed to contain HR cells. All eyes exhibited typical SICS staining when assessed after the confocal assessment.

Table 5-3: Number of hyper-reflective (HR) cells (per eye) of each participant as determined by confocal microscopy in Phase 3: both eyes were exposed to the same lens and solution combination for 2 hours. "n/a" describes cases in which no images were obtainable due to participants' inability to tolerate the imaging procedure. A score of "0" indicates that clear images were obtained and no hyper-reflective cells were observed. *NOTE: Percentage of eyes exhibiting HR cells does not include those "n/a" eyes in which no images were obtained.

ID	RE	LE
1	3	3
2	n/a	6
3	1	4
4	1	3
5	n/a	n/a
6	15	10
7	n/a	4
8	8	5
9	5	6
10	6	10
11	11	9
12	4	6
13	2	10
14	8	7
15	n/a	n/a
16	7	15
17	3	7
18	14	0
19	4	5
20	1	2
# eyes confirmed with HR cells	**16**	**17**
% eyes imaged, with HR cells*	**100%**	**94%**

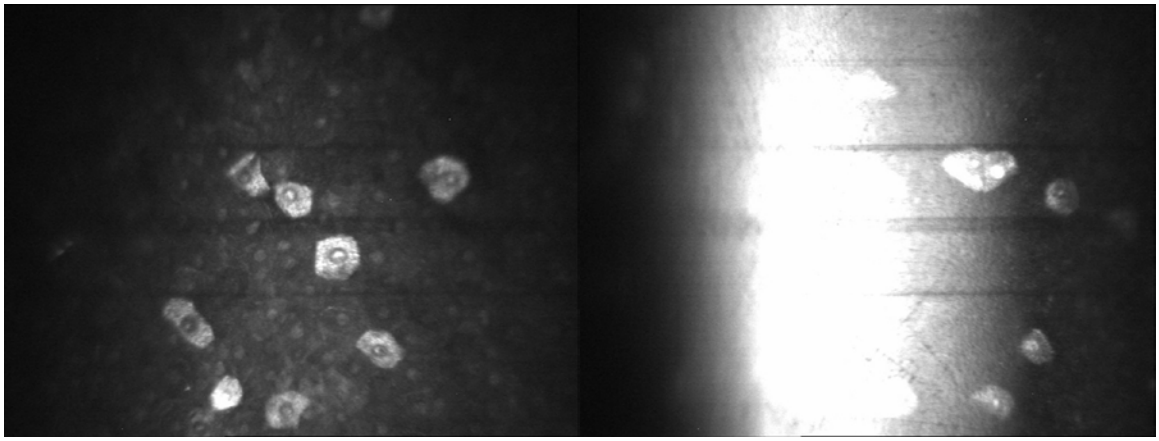


Figure 5-6: Typical image of superficial epithelial cells, showing hyper-reflective cells, in a cornea exhibiting SICS, Phase 3. Left image: Contact lens is in situ, no anaesthetic or fluorescein was instilled; Right image: Contact lens removed, anaesthetic instilled, no fluorescein.

5.5 Discussion

This study confirms that SICS can be induced by wearing PureVision™ lenses that have been pre-soaked in renu® fresh™. Indeed, each of the twenty participants demonstrated SICS, a higher rate of occurrence than reported in some of the earlier publications,[2, 4, 6] although Peterson *et al*.[42] also reported a high response rate (of 80%) with this particular lens and solution combination.

The key objective of the study was to determine whether rinsing the lens or eye would change the area of the SICS response after two hours of wearing lenses soaked overnight in the PHMB-MPS. Rinsing the lens before it was worn had no effect on reducing the area of the cornea exhibiting SICS; there was no difference between the area of corneal staining of the eye that had worn the rinsed lens and the eye that had worn the non-rinsed lens. Similarly, rinsing the eye after lens removal and before corneal staining assessment was made, had no effect on reducing the area of the cornea exhibiting SICS; there was no difference between the area of corneal staining in the eye that been rinsed and the eye that had not been rinsed. In both phases, the mean corneal areas of staining in the treated and untreated eyes were high and at >85%, and thus would be considered clinically relevant. Future studies should give consideration to the effect size that would be meaningful to their experiment. For this pilot study, given the mean staining areas and the standard deviation recorded, a difference of just

9% would have given rise to a statistically significant result, however, it is not likely that reducing the staining level from say 85% to 76% would be considered clinically relevant.

Therefore, the mechanism that causes the appearance of superficial punctate corneal staining in SICS is not affected by copious rinsing of either the lens or the eye. This finding does not rule out the causative mechanism being a leaching of PHMB out of the lens, which subsequently binds to the corneal epithelium. Rather, it infers that if this is the mechanism behind SICS, then the PHMB is not easily rinsed out of the lens with saline and/or that any PHMB which has adhered to the epithelium is resistant to being rinsed off with saline following lens removal.

A study published by Peterson *et al*.[42] showed that using a rub-rinse step before overnight lens storage caused a significant reduction in the SICS appearance. The authors noted that this finding was difficult to explain but they proposed that the rub-rinse may have caused a change in the surface properties of the lens, which in turn changed the uptake and release profile for PHMB.

In this current study, the pan-corneal SICS presentation was more common than peripheral annular staining with a clear central corneal zone. There have been mixed reports regarding the typical presentation of SICS, with some reporting annular staining with central corneal sparing, some reporting pan-corneal staining (which was either evenly distributed or more dense closer to the limbus) and others reporting a mixture of both patterns.[2, 4, 6, 7, 18, 42-45] Given the small sample size of this study, it is possible that this group of participants may not be fully representative of the contact lens wearing population. A much larger incidence study is required to provide more conclusive evidence of the relative distribution of staining patterns among SICS responders. It is also possible that the pattern of the staining is not simply subject dependent, but that the patterns interchange with observation time. To date, there is no evidence of whether the pattern is repeatable within individuals. Future clinical trials of repeated exposures on the same participants would provide evidence of whether the pattern of staining is repeatable and consistent. Varying the assessment time for repeated study of the same individuals would perhaps help us understand whether staining has a shorter duration in the central zone than in the peripheral zones, thus explaining the reports of a higher incidence of the annular pattern.

The evidence of punctate epithelial disturbance under white light suggests that the epithelial cells have already been affected by some mechanism, which may be related to the preservative PHMB. The loss of transparency appears as a punctate pattern where the ‘punctate dots’ are equivalent in size

to individual superficial epithelial cells.[26] This suggests that whatever the SICS response is, it is occurring at the cellular level.

Phase 3 of this pilot study investigated the presence of HR superficial epithelial cells after wearing the pre-soaked contact lenses. Mocan and Irkec[46] used confocal microscopy to look for HR superficial epithelial cells among three subject groups. Before the instillation of fluorescein, they reported incidences of 0% in normals, 17% in those with keratoconus and 40% in those with overt epitheliopathy.[46] In this study, HR epithelial cells were confirmed in most (97%; 33 of 34 eyes) of the participants imaged, a much higher incidence than that observed in damaged corneas by Mocan and Rikec. Topical anaesthetic is known to cause corneal staining in some people, though any confounding effect of this causing HR cells can likely be excluded because Mocan also used a topical anaesthetic, though a different one to that used in this study. Furthermore, the anaesthetic used in this study was also used by Schneider,[29] who also reported an increase in HR cells in the presence of SICS. In this study the epithelial cells were imaged only in the central region, which tended to be the region with the lowest area of epithelial staining. It is uncertain whether the HR cell count would have been higher if the imaging took place in a region exhibiting higher staining levels. The presence of such HR cells has been previously shown to be associated with the presence of SICS[27, 28, 30] and, as in this study, it has previously been reported that there can be variability in cell count among individuals.[29] It has been suggested that these cells become hyper-reflective because of their apoptotic condition (programmed cell death).[31, 47] Additionally, in animal studies, the cells that exhibit hyper-reflectivity have been shown to be those cells that stain with fluorescein.[30] Their presence in association with SICS appears to provide further evidence of some level of physiological change to the epithelial cells.

It would be reasonable to anticipate that damage to the corneal tissue, particularly peripheral corneal tissue, would give rise to a noticeable increase in limbal hyperaemia due to the blood vessel dilation component of the wound healing response. It is therefore surprising that the presence of punctate staining seen in this study, over such large areas of the corneal surface in both eyes, does not appear to be associated with any major change in the limbal hyperaemia from the pre-lens wear levels. The lack of such a response would suggest that either there is no trigger for the healing response when SICS is caused, or that the trigger is somehow blocked by the mechanism causing SICS.

An absence of symptoms has been used to support the PATH theory that SICS is a harmless phenomena of molecular binding to the corneal epithelial surface.[21] Although SICS has not been associated with extreme levels of discomfort, some clinical trials have reported reduced comfort and/or increased dryness[4, 9, 14, 15] while others have reported no affect on comfort.[2, 13] This study has shown that while some participants experienced reduced comfort and specific symptoms in conjunction with SICS, others were asymptomatic.

This study reports a statistically significant drop in comfort ratings between the pre-insertion rating and the post-removal comfort of both eyes in both phases. This comfort drop is also arguably clinically relevant at ≥9 points on the 100-point scale. However, because both treated and untreated eyes exhibited a SICS response, there is no non-lens wearing control to identify whether the reduced comfort was due to the contact lens or the corneal staining response.

The area of corneal staining was fairly consistent across participants, but the comfort ratings data show considerable variation; some individuals reported ratings below 60/100, while others rated their comfort above 90/100. Comparing subjective ratings between individuals can be misleading, however the variability in the comfort ratings contrasts with the consistency of the areas of corneal staining. Although reduced comfort has been associated with SICS, the high variability of the comfort scores have prevented correlations with staining extent to be found.[1, 2, 4, 6, 9, 10, 14, 15] It seems that there may be a factor other than the corneal disruption which is responsible for symptoms in certain individuals. The 'burning' and 'stinging' sensations reported in this study upon lens removal are difficult to explain. It is possible that the lens was acting as a 'bandage' lens while in situ which led to immediate discomfort following removal, though to date this has not been investigated. The previously mentioned literature only reports comfort ratings while the lenses were in situ on the eye, and did not investigate symptomology upon lens removal, as was done in this study.

This pilot study has a number of potential issues in its design that could be addressed in future studies investigating the SICS phenomenon. The study participants were all female and although Young *et al*.[48] showed no association between gender and SICS, it is unknown whether enrolling only a single gender may bias the results. A limitation of the study design is its contralateral comparison, which relies on the two eyes having a similar SICS response. Using a contralateral design in SICS studies was supported by the results of Luensmann *et al*.[49] and randomisation of the treatment eye was used to help mitigate any bias of unequal response between eyes. Phase 3 can be criticised for not having a comparison or control phase to assess HR cells in the participant when no lens was worn.

The only comparison for these findings of high incidence is the result from another report,[46] which suggests that normal healthy corneas do not commonly exhibit HR cells.

This study has demonstrated that rinsing the lens before wear or rinsing the eye after lens removal has no effect on the area of the cornea exhibiting SICS. Historically and intuitively, corneal staining this widespread across the cornea has been regarded as "corneal damage", which typically co-exists with both discomfort and clinically significant limbal hyperaemia, neither of which were evident in this study. The clinical appearance of epithelial disturbance across such large areas of the cornea, both with and without sodium fluorescein, does not seem to be in sync with the lack of associated hyperaemia and the few symptoms. Recent review papers have highlighted how little we actually understand about the role of sodium fluorescein as a diagnostic tool in eyecare.[24, 34, 35] Furthermore, evidence has shown that the epithelial disturbance of SICS will reach a peak and will then show signs of resolving despite continued lens wear.[4] There have been no specific investigations into whether repeated days of wear when SICS is present will cause an increase in signs or symptoms, although Jones *et al.*[2] reported that staining levels were similar after a 2-week and a 4-week exposure to SICS-inducing products. The exact mechanism of corneal staining in general, as well as specifically in SICS, remains unclear and requires more investigation. It is important to understand what is happening at the cellular level in SICS in order to draw conclusions about the long-term implications for ocular health and more work is required in this area to provide some fundamental clues to its etiology.

Chapter 6
SICS: Investigating the Staining Pattern and Repeatability

6.1 Introduction and purpose

As described in Chapter 1, the cause of solution induced corneal staining (SICS) is still being debated,[1, 2] and the physiological implications of the epithelial staining involved are not fully understood.[3-5] Previous investigations have provided sound, repeatable information regarding the products involved and the levels of staining observed. As mentioned in Chapter 1 and in the introduction to Chapter 5, SICS has been shown to be more severe with specific products, including contact lenses of group II hydrogel or silicone hydrogel materials soaked in care products preserved with PHMB or PolyQuad.[6-11] Additionally, the SICS associated with PureVision contact lenses and the two lens care products ReNu MultiPlus and *renu* fresh have been shown to cause the most severe level of SICS of all the product combinations and also shown to have a specific temporal course, with maximal staining occurring between 1 and 4 hours of lens wear.[6, 12] These two care products have an identical chemical composition; ReNu MultiPlus was renamed as *renu* fresh and repackaged into a different style of bottle. The term 'Renu' is used throughout this chapter to refer collectively to ReNu MultiPlus and *renu* fresh lens care products.

Despite so many studies being reported, the typical presentation of this corneal staining phenomenon requires more investigation. There is consensus that the staining is punctate in nature, however how widespread this staining is across the cornea remains in question. In one of the first reports in 2002 by Jones *et al*.[13], SICS was described as a peripheral annulus of staining close to the limbus, typically being that shown in Figure 6-1. Many reports of SICS since have also described the staining as a peripheral annular pattern (also described as a "donut-ring" pattern), with central zone staining being either absent or occurring at a much lower intensity than that seen in the peripheral zones.[6, 14] A few years later, Garofalo *et al*.[12] described SICS presenting in both annular and diffuse "pan-corneal" patterns. Since then, SICS has become known as the donut-ring stain pattern, however the reported data often supports a mix of patterns.[7, 14-19] It has been difficult to explain the reasons for this reporting of minimal involvement of the central zone in the theories explaining SICS. It is unclear whether there is sparing of the central zone or whether the staining in the central corneal zone is simply less severe, or perhaps follows a different time course to the peripheral staining.

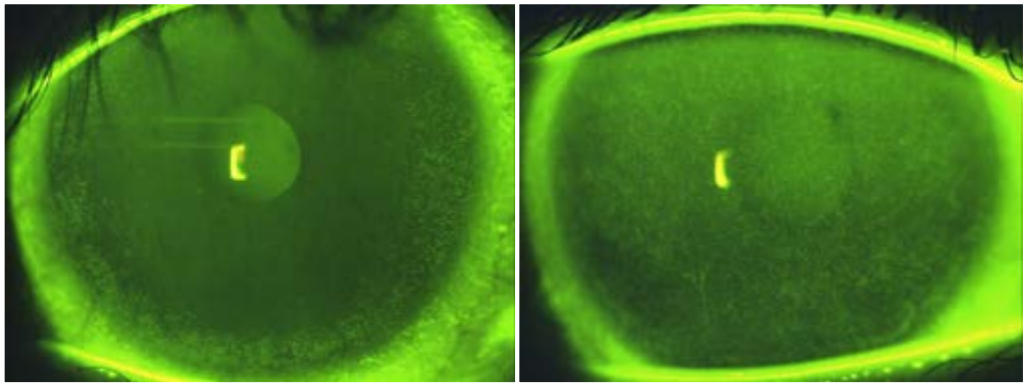


Figure 6-1:The two commonly reported SICS patterns; left image shows the donut-ring pattern, right image shows the pan-corneal pattern.

This chapter reports retrospective data analyses conducted on data from studies conducted at CORE which intentionally induced SICS. These analyses were designed to explore:

- the frequency of the donut SICS pattern relative to the pan-corneal pattern;
- the repeatability of the staining pattern across individuals.

6.2 Study selection criteria

Many studies were reviewed to assess their acceptability for the inclusion of their data in this chapter, Table 6-1. The selection criteria used to determine the inclusion of studies were:

1. Received ethics approval from a University of Waterloo Research Ethics Committee;
2. Was conducted in accordance with the tenets of the Declaration of Helsinki;
3. Conducted a documented informed consent procedure with all subjects;
4. Recruited healthy participants;
5. The balafilcon A contact lens (PureVision, Bauch & Lomb) was worn in combination with a Renu brand PHMB-preserved care product for 1-3 hours;
6. Corneal staining was observed using blue light and a yellow barrier filter;
7. Corneal staining type and staining extent were graded using the 0-100 CORE corneal staining scale, and each of the five corneal zones were graded separately;
8. Lens wear-time was captured at the time of grading corneal staining.

Table 6-1: Details of studies or study arm meeting the inclusion criteria for analysis.

Study code	Sample size	Design; products	Wear time (WT)
A	24	Randomized, double masked, 1mth crossover with washout. Balafilcon A lenses (as per subject prescription) presoaked for >12hrs with ReNu MultiPlus & others. Only data from ReNu MultiPlus arm included.	WT not controlled but captured. Only data with reported WT of 1-3hrs was included in the analysis.
B	35	Non-dispensing, randomized, double masked. -0.25 power balafilcon A lenses presoaked for >12hrs with *renu* fresh & another, contralateral wear. Only data from *renu* fresh eyes included.	WT 2hrs
C	14	Non-dispensing, randomized, double masked, crossover. -0.25 power balafilcon A lenses presoaked for >12hrs with ReNu MultiPlus & others, contralateral wear. Only data from ReNu MultiPlus eyes included.	WT 15min,30min,1hr & 2hrs Only data from the 2hr WT arm was included in the analysis.
D	20	Non-dispensing, randomized, double masked. Bilateral wear of -0.25 power balafilcon A lenses presoaked for >12hours with *renu* fresh. Phase 1 & 2 - one eye received rinsed lens/eye. Only data from non-rinsed eyes included. Note: IDs 1-5 were selected as SICS +ve responders.	WT 2hrs
E	6	Non-dispensing; randomized, double masked. Balafilcon A lenses presoaked for >12hours with ReNu MultiPlus & other solution, contralateral eye design. Only data from ReNu MultiPlus eyes included. All subjects were selected as SICS +ve responders	WT 2hrs
F	5	Non-dispensing; randomized, double masked. Phase 1: balafilcon A lenses presoaked for >12hours with ReNu MultiPlus & other, contralateral eye design. All subjects were selected as SICS +ve responders	WT controlled to 2, 4 and 6hrs. Only data from the 2hr WT arm was included in the analysis.
G		Phase 2: Non-dispensing; randomized, double masked. Balafilcon A lenses presoaked for >12hours with ReNu MultiPlus & other solutions, contralateral eye design. Fluorets and liquid fluorescein randomized. Only data from ReNu MultiPlus eyes and fluoret fluorescein source included. All subjects were selected as SICS +ve responders	WT 2hrs
H		Non-dispensing, randomized, double masked. Balafilcon A lenses presoaked for >12hours with ReNu MultiPlus & other solutions, contralateral eye design. All subjects were selected as SICS +ve responders	WT controlled to 2hrs

6.2.1 Study products

In all studies the contact lens worn was the same brand, PureVision (Bausch & Lomb, NY), Table 6-2. This silicone hydrogel lens was pre-conditioned prior to wear by soaking in one of two Renu care products (Table 6-3) for a minimum of twelve hours prior to being placed onto the eye. The lens cases used for the pre-wear soaking also underwent pre-conditioning in all studies. They underwent seven consecutive twelve hour periods of being filled with the Renu product, replaced daily, immediately before the lens pre-soaking step. The wearing of PureVision lenses pre-soaked in a Renu care product was used as a 'SICS-inducing treatment' in the studies of Table 6-1.

The two Renu lens care products used in these studies, described in Table 6-3, are in fact of identical chemical composition. The change of name to *renu* fresh was made as a change in packaging was introduced. ReNu MultiPlus remained available in the traditional white plastic bottle, while *renu* fresh was packaged in a transparent plastic bottle.

Table 6-2: Details of PureVision contact lens worn in all studies listed in Table 6-1.

Brand name, manufacturer	**Details**
PureVision® Bausch & Lomb, Rochester, NY 	Material: balafilcon A, FDA group V, 36% water silicone hydrogel Dk/t: 99 for -3.00D Parameters: base curve 8.70, diameter 14.0mm

Table 6-3: Details of Renu brand PHMB-preserved products used in the studies listed in Table 6-1.

Brand name, manufacturer	% PHMB	Other ingredients
ReNu® MultiPlus Bausch & Lomb, Rochester, NY 	0.0001	Surfactant/wetting agent: Hydranate 0.03%, Poloxamine 1.0% (Tetronic 1107) Buffer: Boric acid, sodium borate Chelating agent: EDTA 0.1%
renu® fresh Bausch & Lomb, Rochester, NY 	0.0001	Surfactant/wetting agent: Hydranate 0.03%, Poloxamine 1.0% (Tetronic 1107) Buffer: Boric acid, sodium borate Chelating agent: EDTA 0.1%

6.2.2 Staining grading

To be eligible for this analysis, studies were required to record the level of corneal staining using the CORE corneal staining scale. This scale grades the cornea separately in each of the five corneal zones; temporal, superior, nasal, inferior and central. As described more fully in Chapter 2, the CORE corneal staining scale employs 3-factor scoring; type, extent and depth of staining. For SICS, the type of staining is superficial and punctate, therefore the type and depth of the staining has minimal variance. The factor of main interest to describe the variability in the staining presentation is the extent grade, or the area of the cornea exhibiting the staining. The following analyses will report on the extent, or area, of staining.

Punctate staining presents as a region of dots of fluorescein stain and the area of this staining is graded by estimating the area within the border of this region of dots. An example is presented in Figure 6-2, where the eye exhibits a region of punctate staining in the inferior zone. The blue line

outlines the region of staining to indicate the region on the zone affected by the staining. In this example it is estimated to be 22% of the area of the inferior zone.

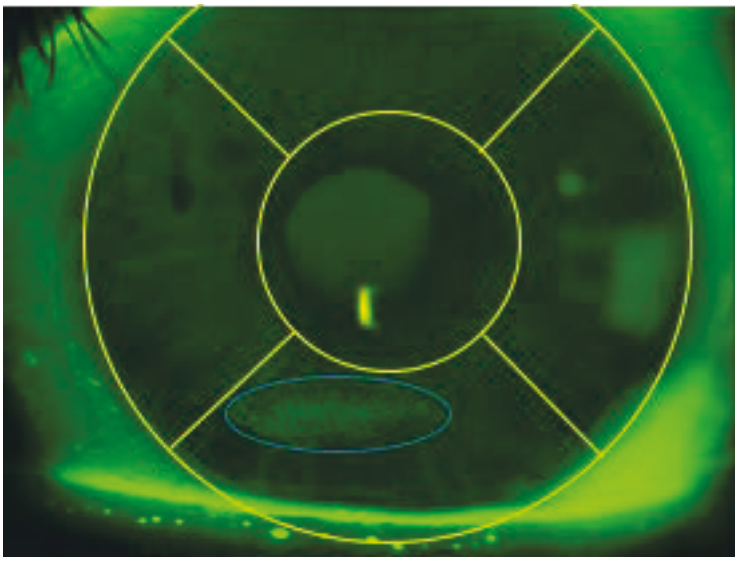


Figure 6-2: The blue line defines the outer border of the area of punctate staining, inferior zone.

6.2.3 Participant inclusion/exclusion

In some studies, participants were specifically invited to take part because they were known to have exhibited a SICS response in a previous study, Table 6-1. These subjects were only included once in the pattern analysis to avoid potential bias. In these instances, the chronologically earliest study data was used.

Study A was conducted over 1-month with follow-up visits at 2-weeks and 4-weeks, but the study design did not control the wear time at these follow-up visits. Fortunately, the actual wear time was captured in the data set. The corneal staining data from the 2-weeks follow-up visit was included in the analysis only if their reported wear time at that visit was between 1 and 3 hours, inclusive. If the subjects' 2-week data was not eligible for inclusion based on the reported wear time, then the lens wear time at their 4-week visit was reviewed and data from this visit was included if the reported wear time at that visit was between 1 and 3 hours, inclusive. Choosing to include data where the contact lens wear time was 1-3 hours is supported by Garofalo *et al*.,[12] who reported that maximum

SICS staining was maximal after 1 hour, minimal at 6 hours and also by work published by Andrasko *et al*.[8] who reported higher levels of SICS after 2 hours of lens wear compared to 4 hours.

For the repeatability analysis, subjects were included if they had participated in three or more of the studies listed in Table 6-1. There was no requirement for them to exhibit a specific level of SICS response in order to be included in this analysis. Several studies recruited participants based on the knowledge that they had exhibited SICS previously, because the presumed likelihood of repeating the SICS response was desirable in order to test the hypothesis.

6.3 Analysis of staining patterns: donut-ring versus pan-corneal staining

6.3.1 Objective

To investigate which corneal staining pattern is the most common SICS presentation, by analyzing the frequency of the donut-ring and pan-corneal patterns.

6.3.2 Methods

Four of the studies listed in Table 6-1 met the inclusion criteria for this pattern analysis studies, providing a sample size of 88 subjects, Table 6-4.

Study A methodology did not control for contact lens wear time at the study visits, however lens wear time at the time of the visit was collected. As described in Section 6.2.3, only those participants who attended their study visit between 1 and 3 hours after lens insertion were included in the analysis. This range of wear period was based on work by Garofalo *et al*.,[12] who reported maximal staining between 1 and 4 hours of wear, and Andrasko *et al*.[8] which supports highest level of SICS after 2 hours wear, which was at a reduced level after 4 hours lens wear. Twenty-four subjects met this criterion.

Study B controlled for lens wear time in the study design, therefore all 35 subjects were included in this analysis.

Study C assessed staining after 4 time periods of lens wear but only the data collected at the 2 hour wear period was included in this analysis.

Study D selected the first five participants from Study B in order to have known SICS responders among the cohort. To avoid repeated inclusion of participants, data from the first five were not included in this pattern frequency analysis, leaving a sample of 15 eligible to be analysed.

Table 6-4: Studies providing data for pattern analysis.

Study code	Sample size included
A	24
B	35
C	14
D	15
Total	88

The type of staining in SICS is generally superficial and punctate in nature,[13, 15] therefore the type and depth of staining shows little variability. The staining variable analysed to investigate the frequency of staining pattern was the area of staining grade for each zone. For studies with bilateral SICS-inducing treatment where data was eligible from both eyes, only the right eye data was included in the analysis, due to the expectation that the staining response would be similar between eyes. Several of the trials were of contralateral design, therefore the data is from a mix of right and left eyes.

An early definition of SICS was provided by Carnt *et al*.[9] in 2007, with reference to the CCLRU grading scale:

> *"... diffuse punctate staining (extent grade 1 and above) in at least four of the five regions (central, superior, inferior, nasal and temporal) of the cornea."*

Grade 1 extent staining was specified in the CCLRU scale as '1-15% surface involvement'. Therefore Carnt's definition specified that diffuse punctate staining in four of the five corneal zones, no matter how small an area was affected, could be termed SICS. This seems to be a very broad staining area criteria, which would define SICS in subjects with very little staining.

A few years before Carnt's definition was published, Garofalo *et al*.[12] presented SICS data from their experiment in several ways; the mean staining area per zone, the mean number of zones affected by staining and the mean number of zones displaying ≥10% area staining. The retrospective analysis described below, which combines data from the four studies listed in Table 6-4, presents the corneal staining data in a manner similar to that used by Garofalo *et al*.[12]

6.3.3 Results

The mean staining area grades, by zone and by study, are presented in Table 6-5 for all subjects, irrespective of whether they presented with SICs. The average staining area for the entire cornea was calculated as the average of the mean areas of the five zones, for all eyes. This value was 65%., indicating that on average each eye across all studies exhibited punctate staining covering 65% of the entire cornea. Figure 6-3 is a representative illustration of this grade of total corneal staining area.

Table 6-5: Mean area of staining by zone (standard deviation) and range, per study, n=88 (0-100).

Study (n)	Temporal	Superior	Nasal	Inferior	Central
A (24)	34 (41)	28 (24)	32 (41)	38 (41)	25 (38)
B (35)	75 (27)	68 (25)	78 (21)	73 (23)	51 (29)
C (14)	67 (38)	63 (41)	67 (39)	69 (34)	69 (36)
D (15)	93 (14)	91 (13)	93 (9)	91 (12)	97 (5)
Mean (SD), range	66 (38) 0-100	60 (38) 0-100	66 (37) 0-100	66 (34) 0-100	54 (39) 0-100

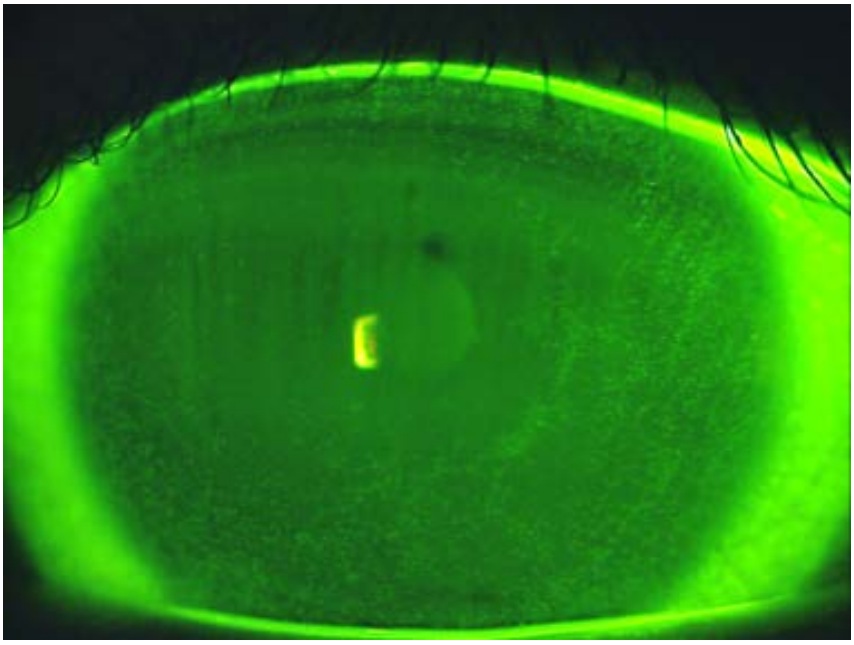


Figure 6-3: Example of punctate staining over approximately 65% of the corneal area.

Applying the Carnt *et al*.[9] definition of SICS to this multi-study data (diffuse punctate of 1% or more extent across at least four zones), 74 of the 88 eyes (84%) exposed to the balafilcon A lens and PHMB care product exhibited SICS. Table 6-6 presents the count of participants who were considered to exhibit SICS when applying the slightly tighter definition based on the paper by Garofalo *et al*.,[12] which requires the staining to be at least 10% of the zone area in four out of five

zones. With this definition, the total number of eyes exhibiting SICS reduced to 71 out of the 88 eyes (81%).

It was of interest to investigate the proportion of these 71 SICS responders that exhibited a donut-ring staining pattern, given that this had been a common description of SICS. The SICS definition chosen for this proportional analysis was based on that first suggested by Garofalo *et al*.[12] such that donut-ring pattern was defined as all four peripheral zones exhibiting staining of ≥10% with either no staining or <10% staining area in the central zone. This count is presented in the third column of Table 6-6 and, out of the 71 participants with SICS, the donut-ring pattern was only evident in 5 cases, which represents just 6% of participants. The pan-corneal staining pattern, where all zones exhibited ≥10% staining, was by far the most common, exhibited by 63 eyes, representing 89% of the SICS cases and 72% of all eyes.

Of the SICS responders, the majority exhibited ≥10% staining in all five zones and would be considered to exhibit pan-corneal staining. There were just three exceptions, and in each of these it was the superior zone that did not meet the staining criteria of being ≥10%. In one of these cases the superior zone showed zero staining and in the other two it showed just 5% staining.

Table 6-6: Count of eyes with: a) SICS: defined as 4 zones with ≥10% staining area; b) donut-ring SICS: defined as central zone has <10% staining area; c) pan-corneal SICS: defined as all 5 zones with ≥10% staining area.

Study (n=)	# (%) with SICS ie. at least 4 zones ≥10% area	# donut-ring pattern ie. CENTRAL zone <10% area	# pan-corneal pattern ie. ALL 5 zones ≥10% area	# undefined SICS pattern
A	9	1	6	2
(24)	(38%)			• 1 eye exhibits 4 zone SICS: S=5% area; • 1 eye exhibits 4 zone SICS: S=0% area
B	35	3	31	1
(35)	(100%)			• 1 eye exhibits 4 zone SICS: S=5% area.
C	12	1	11	0
(14)	(86%)			
D	15	0	15	0
(15)	(100%)			
Total	71	5	63	3
(88)	(81%)	• 7% of all SICS • 6% of all eyes	• 89% of all SICS • 72% of all eyes	• 4% of all SICS • 3% of all eyes

S = superior zone

*all values rounded to the closest integer

To examine the zone most likely to exhibit low levels of staining in the presence of SICS, as defined by Garofalo *et al*.,[12] a count was made, for each study, of the number of each corneal zone that showed <10% staining, Table 6-7. In the 71 eyes exhibiting SICS, of the 355 individual zones across all eyes, only 8 zones exhibited staining of <10% area; five were the central zone and three were the superior zone.

Table 6-7: Using the SICS definition of 4 zones or more with ≥10% area staining, count of corneal zones exhibiting <10% staining area in eyes exhibiting SICS, by study and for all studies combined, n=71.

Study (n)	Temporal	Superior	Nasal	Inferior	Central
A (9)	0	2	0	0	1
B (35)	0	1	0	0	3
C (12)	0	0	0	0	1
D (15)	0	0	0	0	0
Total (71)	0	3	0	0	5

For those participants with this '≥10% definition' of SICS, the mean area grade of the staining in each zone, by study and overall, are shown in Table 6-8. The standard deviation was fairly high in all zones across all studies; study C showed the lowest standard deviation values across all zones. Among these SICS responders of all studies, the mean zone staining was 75%. This value also represents the mean overall corneal staining area across all SICS responders. An observation of punctate staining over 75% of the cornea would be of significant clinical relevance. Figure 6-4 is a representative illustration of a grade of 75% for the total corneal staining area. While the mean area of the central zone was the lowest, it would not be considered a negligible level at 65%.

Table 6-8: Using the SICS definition of 4 zones or more with ≥10% area staining, mean area of staining (standard deviation) per corneal zone in eyes exhibiting SICS, by study and for all studies combined, n=71.

Study (n)	Temporal	Superior	Nasal	Inferior	Central
A (9)	82 (24)	72 (41)	79 (23)	86 (21)	64 (36)
B (35)	75 (27)	68 (25)	78 (21)	73 (23)	51 (29)
C (12)	71 (36)	73 (35)	76 (34)	77 (29)	77 (31)
D (14)	93 (14)	91 (13)	93 (9)	91 (12)	97(5)
Mean (SD), range	79 (27) 10-100	74 (28) 0-100	81 (23) 15-100	79 (23) 20-100	66 (33) 0-100

*all values rounded to the closest integer

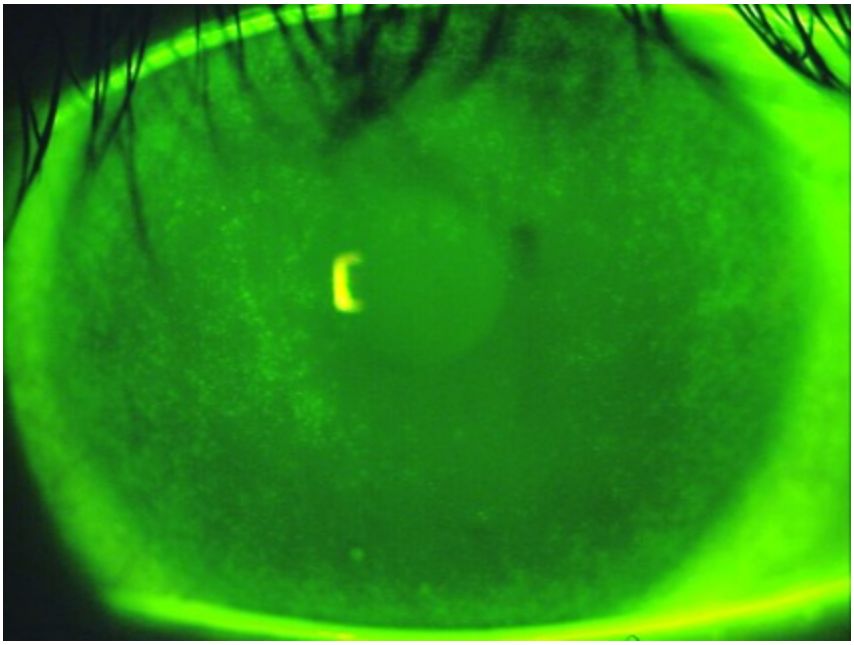


Figure 6-4: Example of punctate staining over approximately 75% of the corneal area.

The data from the eyes exhibiting SICS were manipulated to identify which zone exhibited the highest extent of staining per eye. To achieve this, the zone or zones with the highest extent were allocated a Rank-1 label. For example, if the nasal and temporal zones were graded 75 and the other three zones were grade 50, then both the nasal and the temporal zones would be allocated as Rank-1 for that eye. However, if the nasal zone was graded 75 and the other four zones were graded 50, then only the nasal zone would be allocated Rank-1 for that eye. The number of Rank-1 labels per zone were then summed to show which zone more frequently exhibited the highest area of staining, Table 6-9. The data revealed that the central zone is least likely to exhibit the highest extent of corneal staining in SICS, with a count of 27 (39%). The second least likely was the superior zone with a count of 29 (41%).

Table 6-9: Using the SICS definition of 4 zones or more with ≥10% area staining, count of Rank-1 labels allocated to each corneal zone in eyes exhibiting SICS, by study and for all studies combined, n=71.

Study (n)	Temporal	Superior	Nasal	Inferior	Central
A (9)	4	5	4	7	4
B (35)	21	10	24	15	3
C (12)	6	7	8	8	8
D (15)	6	5	5	5	7
Total count	41	29	45	36	27
& percentage	59%	41%	64%	51%	39%

It may be argued that using 10% as a minimum staining in four corneal zones to define SICS is too low to indicate clinical relevance. Jones *et al*.[13] published one of the first reports of SICS and they proposed that punctate staining that affected close to 50% of the corneal area would be deemed unacceptable and akin to a solution toxicity reaction. Considering this description of clinical significance, the data were reviewed a second time, this time applying a more strict definition of SICS: at least four zones exhibit a minimum of 50% area staining ie. minimum of 40% of the corneal area affected. Under this more strict definition, the count of eyes exhibiting SICS reduced to 59, or 67%, of the full cohort of 88. Albeit a slightly reduced number, this strict definition categorizes two thirds of eyes with significant corneal staining in response to this particular contact lens and lens care product combination.

Data are presented in Table 6-10 using this more strict SICS definition, and the totals across all studies for the donut-ring and pan-corneal patterns have been recalculated. The pan-corneal pattern remained far more common than the donut-ring pattern, at 81% versus 15%.

Table 6-10: Using the SICS definition of 4 zones or more with ≥50% area staining, count of eyes with: a) SICS: defined as 4 zones with ≥50% staining area; b) donut-ring SICS: defined as central zone has <50% staining area; c) pan-corneal SICS: defined as all 5 zones with ≥50% staining area.

Study (n)	# (%) with SICS ie. at least 4 zones ≥50% area	# donut-ring pattern ie. CENTRAL zone <50% area	# pan-corneal pattern ie. ALL 5 zones ≥50% area	# undefined SICS pattern
A **(24)**	7 (29%)	1	6	0
B **(35)**	28 (80%)	8	19	1 1 eye exhibits 4 zone SICS: T=25% area.
C **(14)**	9 (62%)	0	8	1 1 eye exhibits 4 zone SICS: T=45% area.
D **(15)**	15 (100%)	0	15	0
Total (88)	59 (67%)	9 15% of all SICS 10% of all eyes	48 81% of all SICS 55% of all eyes	3 5% of all SICS 3% of all eyes

Data describing the distribution of staining is presented in Table 6-11. The counts of zones with <50% staining area was low; nine were the central zone, three the temporal zone. The mean area of staining was fairly evenly distributed, with the lowest value (75% area) in the central zone. The central zone also had the lowest count of Rank-1 staining area (Rank-1 represented the zone/s with the greatest staining area per eye).

For those eyes exhibiting SICS defined as exhibiting ≥50% area staining in four corneal zones, the mean overall corneal staining area, across all studies, was 84%. This value is higher than that suggested by Jones *et al*.[13] as representing significant staining akin to toxicity staining, and would certainly be considered highly clinically relevant in a consulting room examination. Figure 6-5 illustrates this level of corneal punctate staining.

Table 6-11: Across all studies, for eyes exhibiting SICS according to the definition of 4 zones or more with 50% area staining,: a) count of eyes per zone with <50% staining area; b) mean area of staining (standard deviation) per corneal zone; c) count per zone of Rank-1 labels. n=58.

All Studies	Temporal	Superior	Nasal	Inferior	Central
Count <50% staining area	3	0	0	0	9
Mean area (SD), range	89 (15) 25-100	85 (15) 50-100	89 (12) 50-100	87 (15) 55-100	75 (27) 0 -100
Count Rank-1 percentage	40	27	40	32	25

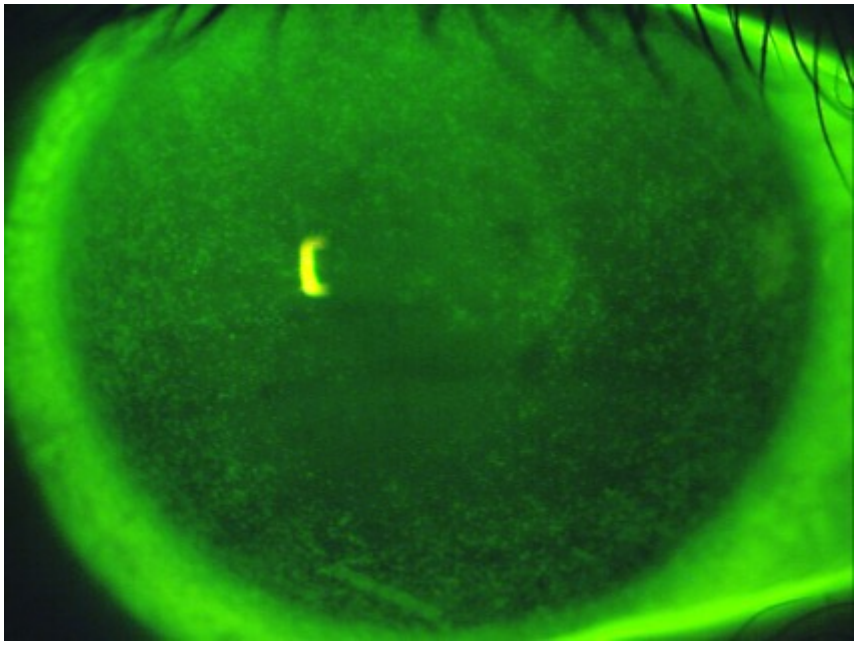


Figure 6-5: Example of punctate staining over approximately 84% of the corneal area.

6.3.4 Discussion

This analysis firstly defined SICS in the same way as previously proposed by Garofalo *et al*.[12]; at least four of the five corneal zones need to exhibit punctate staining over ≥10% area. Of the 88 subjects eligible for this analysis, 71 met this criteria, indicating an incidence of 81%. Even applying

a more strict definition of SICS, which required at least 50% area staining in four of the five corneal zones, two thirds (59) of all eyes still met the criteria. This data supports previous reports that SICS is commonly induced with the combination of balafilcon A contact lens material and one of the Renu, PHMB-preserved care products.[6, 7, 9, 10, 17, 20]

Study A gave rise to lower incidence of SICS than any of the other studies. The methodology in this study employed a rub step to clean the lenses as part of the pre-soaking process. This rub-step has since been investigated by Peterson *et al*.[21] in a group of twenty participants and shown to consistently and significantly reduce the SICS response compared to not using a rub step. The staining data were reported using a combination grade and therefore cannot be directly compared to these results. They proposed potential theories why the rub step made such a difference but no further work was published on this particular aspect. Removing data from Study A because of this difference in contact lens preparation would increase the incidence of SICS found in this analysis. However the main objective was to investigate the frequency of the pattern of SICS, not the incidence of SICS, therefore the sample was included.

No matter which definition of SICS was applied, the vast majority of subjects exhibited pan-corneal rather than donut-ring pattern.

Using the definition of 'four zones ≥10% staining area', 63 (or 89%) of the 71 subjects with SICS showed ≥10% staining area in all five zones ie. the pan-corneal pattern. When analyzing the staining of those subjects with SICS, the lower incidence of staining in the central zone was supported by three methods. Firstly, the count of each zone with <10% area staining was the highest for the central zone at five, and second highest for the superior zone at three. It is of interest to note that none of the other zones showed <10% staining area in any eyes with SICS. Secondly, the mean staining area per zone is lowest for the central zone at 66%. The zone with the second lowest mean staining area is once again the superior zone at 74%, however the other zones follow closely at 79% (temporal and inferior zones) and 80% (nasal zone). The third analysis method employed a staining area ranking system where the zone/s of the highest area of staining per eye were allocated a Rank-1 designation. Once more, the central zone exhibited the lowest count of Rank-1 at 27, with the second lowest count of 29 attributed to the superior zone. For eyes exhibiting SICS according to this definition, while the central zone showed the least staining of all the five zones, it was still present at a clinically significant level, covering on average, 66% of the central zone area.

Applying the more strict SICS definition of 'four zones ≥50% staining area', 44 (or 76%) of the 58 subjects with SICS showed ≥50% staining area in all five zones. As was the case with the less strict SICS definition, all three analysis types supported the central zone as being least affected, though there is still significant staining in this central zone. The count of zones with <50% staining area was nine for central zone, three for temporal and none elsewhere. The central zone had the lowest mean staining at 75%, however, this level is clinically relevant and the other zones were even higher, all >80%. Also, as previously, the count of Rank-1 allocations were lowest for the central zone, followed by the superior zone.

The data for this analysis are derived from 88 individuals who were exposed to the same combination of products, known to induce a SICS response. The fact that SICS was detected was not a surprise, indeed it was expected. The unknown factors were the incidence of SICS and the frequency of donut-ring pattern compared to pan-corneal pattern. Despite the staining pattern being frequently referred to as a donut-ring or peripheral annulus, this study shows the pan-corneal pattern to be by far the most typical presentation. It is unfortunate that the donut-ring term has become synonymous with SICS because it is suggestive of minimal or no involvement of the central zone when, in fact, SICS commonly displays considerable staining in this zone, albeit often slightly less than that in the peripheral zones; 66% or 75% depending on whether the SICS definition is based on 10% or 50% area of staining in four zones.

There are limited comparable data in the literature due to the use of different product combinations or no control over the wearing time before staining assessment was made. However, Andrasko and Ryen[8] used the same product combination among the 59 product combinations they tested. They reported that the Purevision lens, worn in association with the Renu care products, induced some of the highest staining levels after two hours of wear. They also reported the mean overall corneal staining response after two hours to be punctate staining covering 73% area. This value is very close to the results of this analysis, which was 75%. In one of the first reports of SICS, Jones *et al*.[13] also used the same product combination however there was no control over the wear time prior to the staining assessment. Despite not targeting observation at what was later recognized as maximal staining time, 17 of the 44 subjects (37%) presented with staining levels that warranted discontinuation of products. Another observation they made was that the most staining was present in the inferior zone. This current analysis does not support this finding, though Pritchard *et al*.[7] also reported higher staining inferiorly with SICS. They tested an FDA Group II hydrogel contact lens

(alphafilcon A) and reported more corneal staining with ReNu MultiPlus than with either ReNu MultiPurpose or OptiFree Express, which contain different preservatives and other ingredients. Staining in at least three corneal quadrants was reported in twelve out of the 22 subjects, an incidence of 55%.

In the Garofalo *et al*.[12] study, they included the ReNu MultiPlus care product combined with several contact lenses, though they did not use the balafilcon A material. With the hydrogel lenses, ReNu MultiPlus was associated with ≥10% area staining in four or five zones in a maximal staining period of one to four hours of lens wear. With silicone hydrogels they found a lower staining response, with levels of ≥10% area only observed in one or two zones at the maximal staining time of two hours wear time.

The most comment presentation of SICS staining in this analysis was punctate staining over the majority of the cornea - a diffuse pan-corneal presentation pattern. The staining frequently presented over a slightly smaller area in the central zone than the peripheral zones, however, the grade of the central zone staining was still high enough to cause clinical concern. Central staining carries clinical significance because of the impact it may have in the short or long term on vision quality. However, the peripheral corneal regions close to the limbus host the stem cells responsible for replacing the sloughed apical epithelial cells and therefore maintaining corneal barrier function. Therefore, significant and chronic peripheral corneal staining should also be viewed as potentially deleterious to corneal and ocular health. Of the 88 subjects exposed to this product combination, just over half of them, 47 subjects, presented with punctate staining spread, on average, over 84% of their cornea. This extent of punctate corneal staining would certainly be regarded as highly clinically significant and should cause the practitioner to take remedial actions, likely involving temporary cessation of contact lens wear and a change in the contact lens and lens care combination.

6.4 Analysis of staining repeatability

6.4.1 Objective

To investigate the repeatability of the grade of SICS staining extent (area) and the SICS pattern across those participants identified as participating in three or more SICS-inducing experiments at CORE.

6.4.2 Methods

The CORE studies that met the criteria for further SICS data analysis are listed in Table 6-1. All of these studies used the same SICS-inducing treatments, controlled the exposure time to these treatments to two hours and used the same methodology for observing and grading the area of punctate staining. They were further reviewed to identify participants who had participated in at least three studies. This was possible because several of the studies targeted recruitment to those who had been identified as a '*SICS responder'*.

Not everyone participated in every study but the following numeric codes identify the subjects and their repeat participation is shown in Table 6-12. Seven participants were identified as being involved in three or more studies involving SICS-inducing treatments, which spanned a total of six studies, coded C to H, although no participant was enrolled in all seven studies. All of these studies were completed within a period of four years.

The staining data for each subject was compared across the different studies. The data was evaluated for the amount of staining ie. the extent grade, as well as the SICS pattern presented; donut-ring or pan-corneal.

Table 6-12: Subjects identified as being in repeated SICS-inducing studies (n=7).

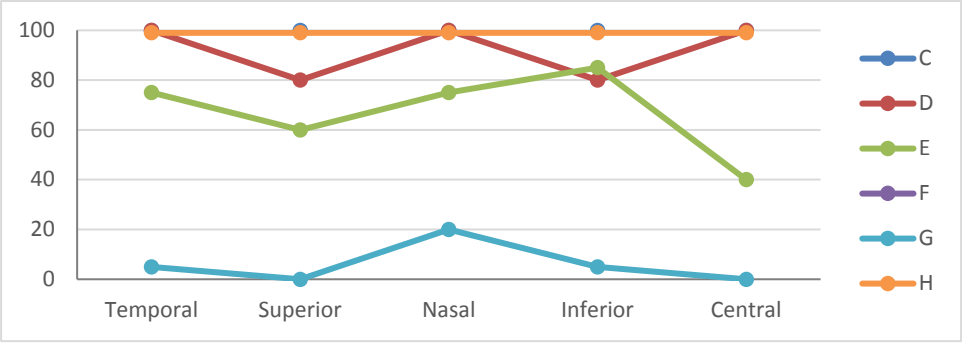
ID	Studies						# repeats
	C	D	E	F	G	H	
1	√	√	√	_	√	√	5
2	√	_	√	√	√	√	5
3	_	√	√	√	√	√	5
4	√	√	√	_	_	√	4
5	√	√	√	_	√	_	4
6	_	√	_	_	√	√	3
7	_	√	√	_	√	√	4

6.4.3 Results

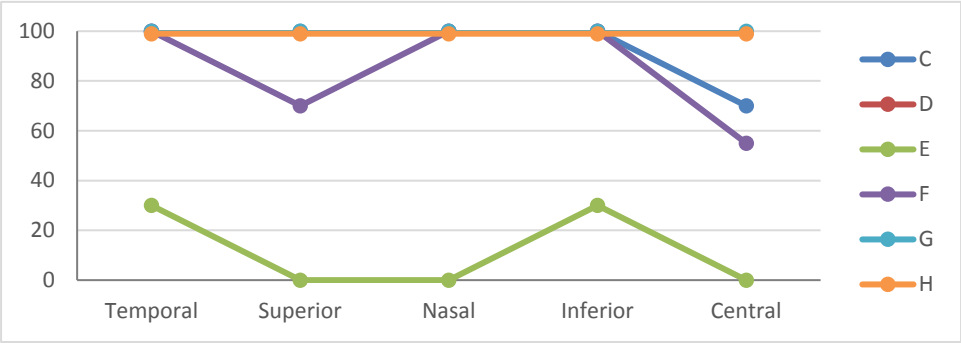
Each participant's data is presented separately to allow review of the intra-participant variability across the specific studies they participated in, Table 6-13. The individual zone staining extent grades are presented as well as the ocular mean staining grade, calculated by summing the zone grades and dividing by five. Calculations of the standard deviation across the individual zone scores of all studies, as well as across the total cornea mean grades of all studies, provides an indicator of variability within the data. With the exception of ID3, the variability of the grades of this 0-100 scale is above 30, which is relatively high. The graphs show the zone staining area grades, colour-coded according to study. This provides a visual illustration of how the SICS response varied across the studies.

Table 6-13: Grades of staining areas arranged by subject, per study and by zone; SICS confirmation and pattern description according to the definitions as specified.

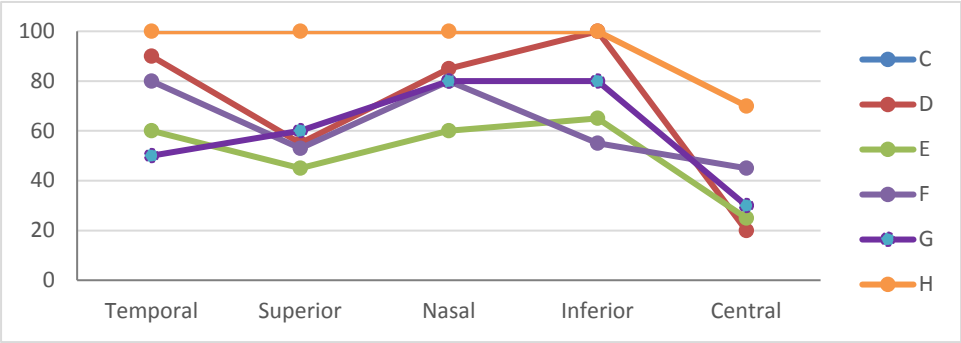
ID 1	**Zone extent grade**						**≥10% in ≥4 zones**			**≥50% in ≥4 zones**		
Study	T	S	N	I	C	Total cornea	SICS	Donut	Pan-corneal	SICS	Donut	Pan-corneal
C	100	100	100	100	100	100.0	√	X	√	√	X	√
D	100	80	100	80	100	92.0	√	X	√	X	-	-
E	75	60	75	85	40	67.0	√	X	√	√	√	X
G	5	0	20	5	0	6.0	X	-	-	X	-	-
H	100	100	100	100	100	100.0	√	X	√	√	X	√
Mean	76.0	68.0	79.0	74.0	68.0	73						
StDev	41.1	41.5	34.7	39.6	46.0	39.8						



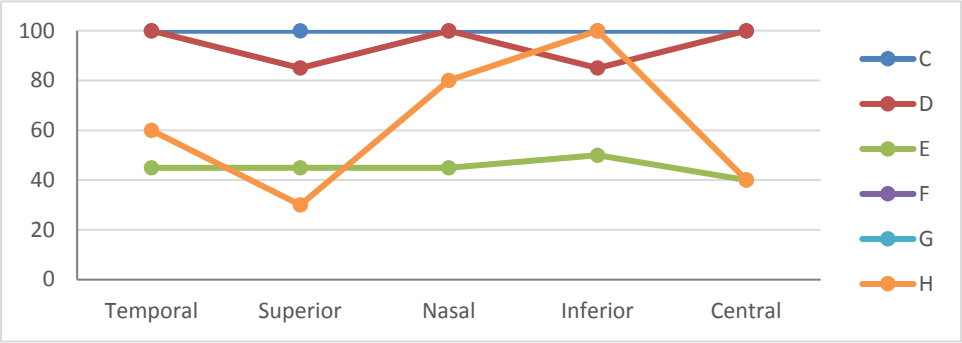
ID 2	Zone extent grade						≥10% in ≥4 zones			≥50% in ≥4 zones		
Study	T	S	N	I	C	Total cornea	SICS	Donut	Pan-corneal	SICS	Donut	Pan-corneal
C	100	100	100	100	70	94.00	√	X	√	√	X	√
E	30	0	0	30	0	12.00	X	-	-	X	-	-
F	100	70	100	100	55	85.00	√	X	√	√	X	√
G	100	100	100	100	100	100.00	√	X	√	√	X	√
H	100	100	100	100	100	100.00	√	X	√	√	X	√
Mean	86.0	74.0	80.0	86.0	65.0	78.2						
StDev	31.3	43.4	44.7	31.3	41.2	37.5						



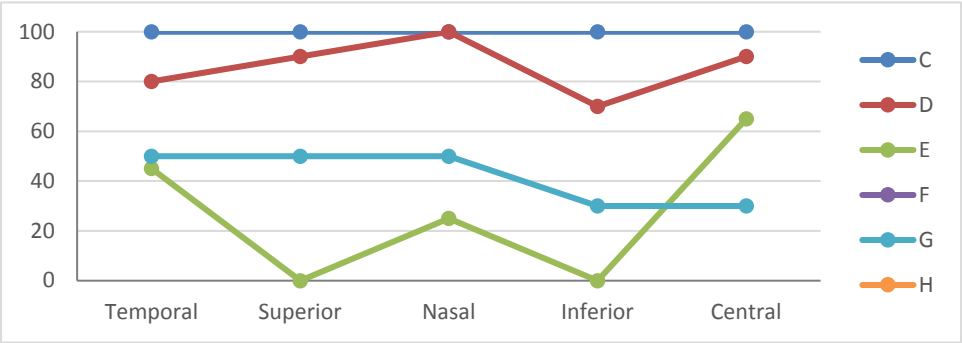
ID 3	Zone extent grade						≥10% in ≥4 zones			≥50% in ≥4 zones		
Study	T	S	N	I	C	Total cornea	SICS	Donut	Pan-corneal	SICS	Donut	Pan-corneal
D	90	55	85	100	20	70.0	√	X	√	√	√	X
E	60	45	60	65	25	51.0	√	X	√	X	-	-
F	80	53	80	55	45	62.6	√	X	√	√	√	X
G	50	60	80	80	30	60.0	√	X	√	√	√	X
H	100	100	100	100	70	94.0	√	X	√	√	X	√
Mean	76.0	62.6	81.0	80.0	38.0	67.5						
StDev	20.7	21.6	14.3	20.3	20.2	16.3						



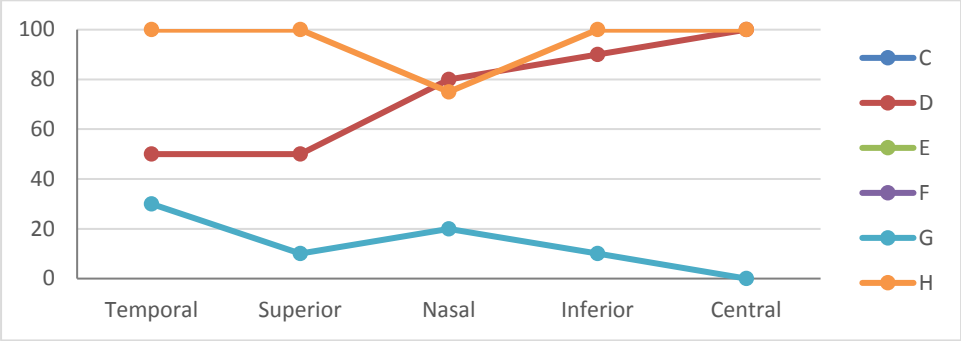
ID 4	Zone extent grade						≥10% in ≥4 zones			≥50% in ≥4 zones		
Study	T	S	N	I	C	Total cornea	SICS	Donut	Pan-corneal	SICS	Donut	Pan-corneal
C	100	100	100	100	100	100.0	√	X	√	√	X	√
D	100	85	100	85	100	94.0	√	X	√	√	X	√
E	45	45	45	50	40	45.0	√	X	√	X	-	-
H	60	30	80	100	40	62.0	√	X	√	X	-	-
Mean	76.3	65.0	81.3	83.8	70.0	75.3						
StDev	28.1	32.9	25.9	23.6	34.6	26.2						



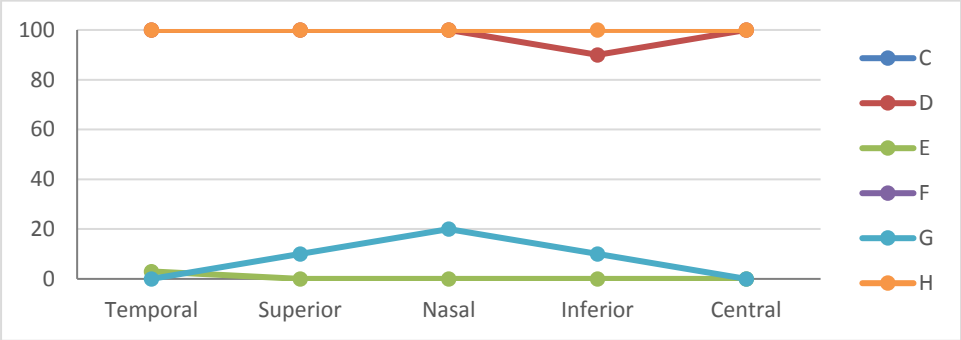
ID 5	Zone extent grade						≥10% in ≥4 zones			≥50% in ≥4 zones		
Study	T	S	N	I	C	Total cornea	SICS	Donut	Pan-corneal	SICS	Donut	Pan-corneal
C	100	100	100	100	100	100.0	√	X	√	√	X	√
D	80	90	100	70	90	86.0	√	X	√	√	X	√
E	45	0	25	0	65	27.0	X	-	-	X	-	-
G	50	50	50	30	30	42.0	X	-	-	X	-	-
Mean	68.8	60.0	68.8	50.0	71.3	63.8						
StDev	25.9	45.5	37.5	44.0	31.2	34.8						



ID 6	Zone extent grade						≥10% in ≥4 zones			≥50% in ≥4 zones		
Study	T	S	N	I	C	Total cornea	SICS	Donut	Pan-corneal	SICS	Donut	Pan-corneal
D	50	50	80	90	100	74.0	√	X	√	√	X	√
G	30	10	20	10	0	14.0	X	-	-	X	-	-
H	100	100	75	100	100	95.0	√	X	√	√	X	√
Mean	60.0	53.3	58.3	66.7	66.7	61.0						
StDev	36.1	45.1	33.3	49.3	57.7	42.0						



ID 7	Zone extent grade						≥10% in ≥4 zones			≥50% in ≥4 zones		
Study	T	S	N	I	C	Total cornea	SICS	Donut	Pan-corneal	SICS	Donut	Pan-corneal
D	100	100	100	90	100	98.0	√	X	√	√	X	√
E	3	0	0	0	0	0.6	X	-	-	X	-	-
G	0	10	20	10	0	8.0	X	-	-	X	-	-
H	100	100	100	100	100	100.0	√	X	√	√	X	√
Mean	50.8	52.5	55.0	50.0	50.0	51.7						
StDev	56.9	55.0	52.6	52.3	57.7	54.8						



6.4.4 Discussion

These seven subjects were invited to participate in repeated studies because they were known to have exhibited SICS with the same contact lens and care products. Depending on the definition of SICS used (four zones with ≥10% or four zones with ≥50%), the number of participants who consistently exhibited SICS across all studies varied from two to none, respectively.

Viewing the participants' data tables, there is little repeatability seen. This was very surprising given the very similar protocols these studies shared. Perhaps the highest level of repeatability is demonstrated by the data of ID3, which has the lowest values of standard deviations. Also, all of their study plots follow a similar path, with all the peripheral zone grades close to or above 50, and all the central zone grades dropping lower. For this participant, all studies led to a confirmation of SICS based on 4 zones ≥10%, however only four of the five studies demonstrated SICS based on 4 zones ≥50%. Conversely, the data plots from ID7 show two distinctly different responses, evenly divided across the four studies; two studies show all zones graded at ≥90%, and the other two show them all graded ≤20%. Similarly, both ID 1 and ID2 show a repeatable response across four of their five studies with high grades (around 70) across all zones, however they each have one other plot that is much lower, with grades around 20. The studies associated with the low grades for these two participants are different, and there is no one study that is consistently associated with the lower grade responses. Conversely, studies C, D and H seem to be consistently associated with high responses, but the reasons for this are unclear.

There are several factors that can increase variability in a subjective clinical measure such as staining grading. One of the obvious potential causes is that the study protocol was not followed properly. This could include the use of incorrect products, errors in the pre-conditioning procedures, or use of non-standard methods for the fluorescein instillation and staining observation methods. Errors in any of these areas has potential to significantly alter the results. This seems unlikely given the experience of the CORE research team and the internal oversight processes. The studies involved several different investigators and their inter-investigator variability could have been a contributing factor. However, while this may explain a difference between grades of 10 or even 20 between studies, it is not likely to be the cause of difference as large as 60 or 80, which is the case for some participants, for example ID7 who exhibits staining areas of close to 100 in two studies, yet in the other two studies exhibits grades of 20 or less.

It is quite plausible that the variability is due to changes within the participant, particularly considering the four-year period over which these studies were conducted. If SICS is influenced by habitual lens wear, environmental conditions, allergy, tear film quality etc then assessments spanning several years may be expected to vary. Health and medications can have a ubiquitous effect on the tear film composition and therefore it is feasible that, even though all subjects reported good health when they were enrolled, some minor fluctuations and changes may have caused the ocular surface response to alter.

Lastly, there is the possibility that the variation stems from an unexpected source, the between eye variability. All but one of the six studies was a contralateral design, therefore the data from the eye exposed to the SICS-inducing treatment eye may have come from the right eye in one study and from the left eye in another study. While it is generally assumed that a physiological response to this kind of stimulus would be similar between eyes, this has never been properly investigated for the SICS response. Also, the level of response may vary according to participant criteria such as tear film quality, which may vary over time and may also be impacted by environmental conditions, General health and medications can impact tear film quality although all participants were healthy at the time of enrollment into these studies, therefore it is anticipated that these impacts will be minimal.

The evidence from this small group of participants unequivocally supports the need for a study designed to specifically investigate the repeatability of the SICS response within individuals. This study should control for as many factors as possible in an attempt to reduce sources of variability. Firstly, the data should be collected within a few months, rather than over years as was the case for this analysis. This would minimize environmental and health variations. Secondly, assigning the same investigator to each participant would remove inter-investigator grading variability, and prior training on the grading scale could be employed to minimize intra-investigator variability. Photographic capture of the corneal staining and/or objective grading software may be valuable additions to the subjective grading, though recent reports have indicated that grading from photographs is quite different to the live situation. Repeated exposure to the same SICS-inducing treatment on at least five occasions would be valuable and increasing the sample size would provide more power to the results, depending on the variability measured. Only when such a targeted study is conducted, can we gain a better insight into the variability of the SICS response among individuals.

Chapter 7
Discussion, Future Work & Summary

This thesis targeted specific aspects of corneal fluorescein staining.

Chapter 1 presents an overview of the importance of maintaining corneal integrity to preserve ocular health and good vision. Because the corneal epithelium provides an important barrier function to protect the rest of the cornea, and therefore the entire eye, then epithelial integrity is also regarded as essential to preserve ocular health and vision. The use of fluorescein to assess epithelial integrity is explained and example images of corneal staining related to dry eye and contact lens wear are included. Though the exact mechanism of corneal fluorescein staining may not be fully understood, it is believed to highlight undesirable physiological responses of the corneal epithelium, and as such requires more study. This thesis provides several pieces of information to further the knowledge in this area.

Chapter 2 provided an overview of the various grading scales to record corneal staining and introduced and described the CORE corneal staining scale. The CORE scale was the method used to record the level of solution induced corneal staining in all the clinical trials featured in Chapters 5 and 6, and most of those assessing dry eye in Chapter 4. A unique feature of this scale is the grading of the 'extent' of the staining and the zonal grading provides the 'spread' of the staining across specific zones, as well as the entire corneal surface. The fact that the extent is graded using a 0-100 scale means it can be interpreted as the percentage of the cornea exhibiting corneal staining, which translates readily to clinical interpretation.

The Chapter 3 described an experiment to assess the agreement of fifteen observers using the CORE corneal staining scale when they graded 22 photographic images at two separate grading sessions. The results unequivocally supported the benefit of prior training, because the one naïve observer demonstrated by far the poorest intra-observer agreement. The results also identified future opportunities to improve the CORE corneal staining scale. Providing pictorial references and clear instructions have both been recommended to be beneficial to all grading scales. The images from the experiment that provided low inter-observer grade variability make good candidates for the development of a pictorial reference guide. These reference images would need to be assigned a grade. However, the mean grade for each image was impacted by outliers and less experienced observers in this experiment. Calculating the mean grade for each image using a bootstrapping

method would reduce the impact of these outliers. The images with high inter-observer variability are valuable for discussions aimed at understanding how the observers approached the grading of these images. Such discussions would expand the instructions on the use of the scale and better instructions may help to further improve agreement. While the 0-100 scale is somewhat straight forward to apply to the *extent* factor of the staining, applying 101 steps to the *type* factor is, anecdotally, reported as more difficult, perhaps because this factor is more descriptive. Further analysis of the results may inform whether the *type* scale would have better agreement with a reduced number of steps, without sacrificing sensitivity.

Chapter 4 evaluated the distribution of corneal staining in 368 subjects with symptoms of dry eye, across 13 studies. Because corneal staining is a common sign used to diagnose dry eye disease, understanding the typical presentation is valuable to the planning of assessment strategies. The analysis demonstrated that in 52.5 %, of all subjects, the inferior zone was the most common location for the greatest degree of staining of the entire cornea. This was true whether the staining occurred only in one zone or whether it affected multiple corneal zones. This means that any dry eye treatment study should monitor the inferior zone staining. Grading the staining over the cornea as a whole may not provide data robust enough to show meaningful change. Also, if the inferior zone is where the worst staining is, then improvements in staining in this zone following any treatment will be most impactful.

Chapter 5 reported on a SICS-inducing experiment involving 20 subjects, which investigated whether rinsing the lens prior to wear and rinsing the eye prior to staining evaluation would eliminate the SICS response. The unilateral rinsing treatment did not eliminate the SICS response, indicating that the rinsing procedure was not able to remove sufficient PHMB, and/or other care solution components, to change the staining outcome. Irrespective of the rinsing treatment, the test and control eyes all exhibited punctate staining over >84% of the entire corneal surface. This study photographed the grey punctate ‘white light staining’ and also imaged hyper-reflective epithelial cells prior to instilling fluorescein. Both of these provide evidence of a cellular response in the absence of fluorescein. Reports of symptoms were collected and though the majority of participants were symptom free, there were unexpected reports of stinging and burning immediately after lens removal. The high levels of staining suggest high clinical relevance while there are contradictory low levels of symptoms and unchanged limbal hyperemia. It appears that SICS may be different to the symptomatic staining associated with dry eye and corneal injury. Future explorations of these

potential differences may involve collecting corneal cells from participants with corneal staining due to dry eye, and comparing them to participants with an induced SICS response and also to cells from healthy corneas. It would be useful to compare morphology across these three groups as well as whether fluorescein penetrates to the cytoplasm or remains solely membrane bound.

Chapter 6 analysed the staining patterns reported across several SICS-inducing clinical trials. The presentation of staining has been frequently reported to occur as a characteristic donut-ring pattern, implying sparing of the central corneal zone. Contrary to this description, the staining in the experiment in Chapter 5 was almost exclusively pan-corneal. Data from several studies that followed the same methodology were combined and analysed. The results showed that, no matter which definition of SICS was applied, pan-corneal staining pattern was far more common than a 'donut-ring' pattern with central zone sparing. Pan-corneal pattern was present in 89%, or 81% of SICS exhibiting eyes, depending on whether the definition of SICS applied was ≥10%, or ≥50% staining extent in four of the five corneal zones. An additional analysis was conducted to investigate the repeatability of the SICS response within individuals. This was a small sample of only seven participants and the results were contrary to expectations; the SICS response among individuals was not predictable. There is a general assumption for subjects recruited into such studies that 'once a SICS-responder always a SICS-responder', and this result challenges this assumption. The small sample size and the fact that the assessments are spread across different clinical trials spanning several years may all be confounding factors. A better way of assessing repeatability of SICS response would be to conduct a project specifically targeted for this purpose. Such a project would likely involve five or more repeated SICS-inducing treatments, per participant. Assigning the same investigator to conduct all the assessments would be of benefit to avoid inter-observer variability. Collecting photographs of the staining would also be valuable.

Summary

For an assessment technique that has been used for over a century, it is quite remarkable that we still do not fully understand how fluorescein interacts with the corneal epithelial cells. This thesis has expanded knowledge in certain aspects of this broad topic.

The CORE corneal staining scale includes an *extent* grade which provides unique data on the percentage area of the cornea affected. This information can be particularly useful in dry eye and contact lens clinical trials. Further development of this scale will create a valuable corneal staining assessment tool.

This thesis provided evidence that the most severe corneal staining in patients with symptoms of dry eye most often presents in the inferior zone. This information highlights the importance of specifically assessing inferior zone corneal staining in future research reporting the efficacy of dry eye treatments.

The SICS-inducing experiment demonstrated that lens rinsing prior to wear, and ocular rinsing prior to staining evaluation, do not eliminate the SICS response. Additionally, the presence of 'white light' staining and hyper-reflective cells in almost every SICS case further supports other reports of cellular changes being present prior to the instillation of fluorescein. The multi-study analyses demonstrated that the appearance of SICS was more common as a pan-corneal distribution, rather than the frequently reported 'donut-ring' pattern. Analysis of individuals exposed to the same SICS-inducing treatment in different clinical trials raised questions about the repeatability of the extent and pattern of staining observed. A targeted investigation would be valuable.

Corneal fluorescein staining has been the subject of much study in recent years, particularly since the first reports of SICS. While much has been learned there are still many areas that require more investigation. In particular, understanding the cell transport mechanisms involved in fluorescein staining through improved *in-vitro* or *ex-vivo* techniques, and the development of higher magnification *in-vivo* cell imaging equipment may provide the much needed answers to questions of clinical relevance.

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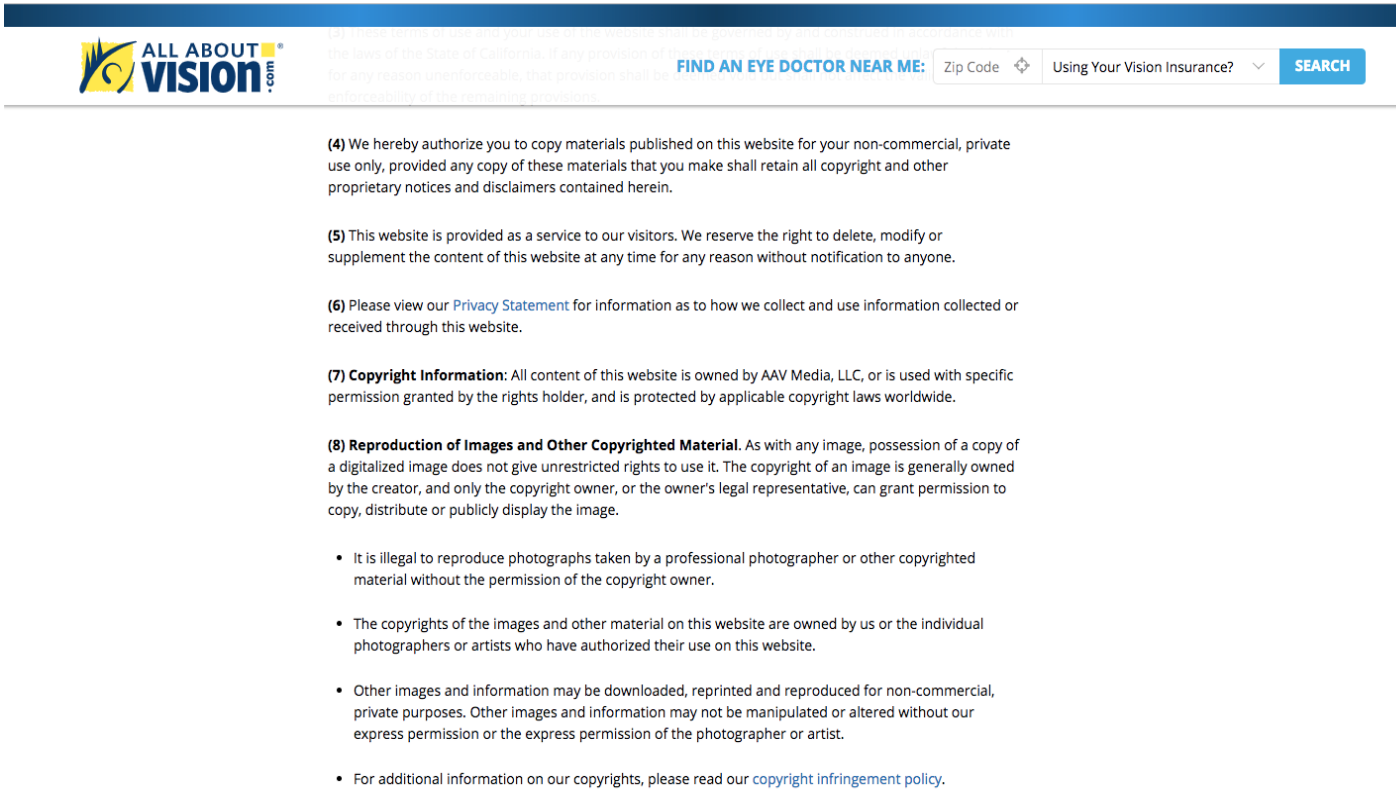

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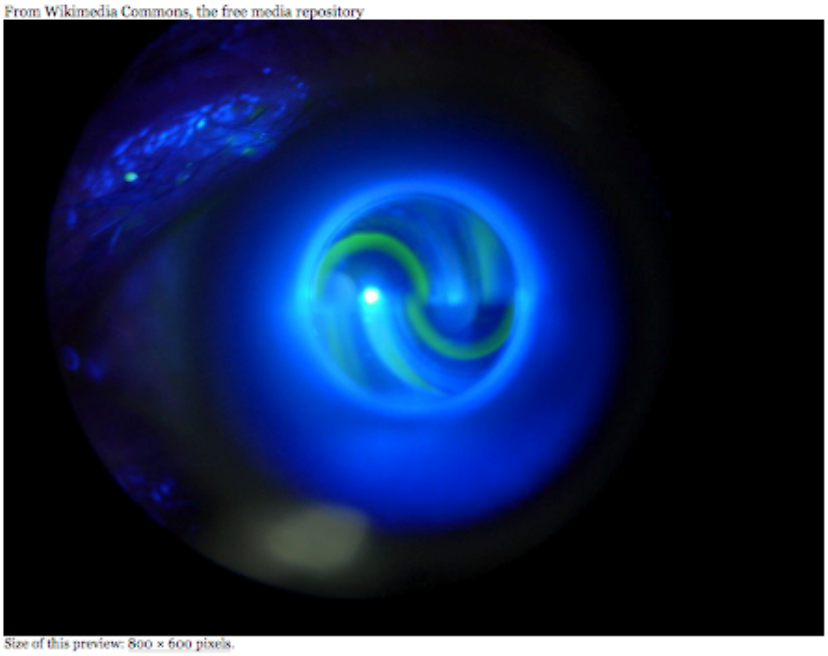

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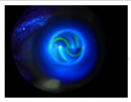
current	**18:07, 14 February 2009**		2,592 × 1,944 (1.01 MB)	File Upload Bot (Magnus Manske) (talk | contribs)	{{BotMoveToCommons|en.wikipedia}} {{Information |Description={{en|0}} |Source=Transferred from [http://en.wikipedia.org en.wikipedia]; transferred to Commons by User:Roberta F. using [http://tools.wikimedia.de/~magnus/commonshelper.php CommonsHelper]

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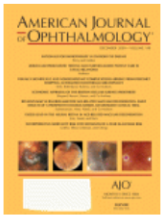

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CHAPTER 1

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