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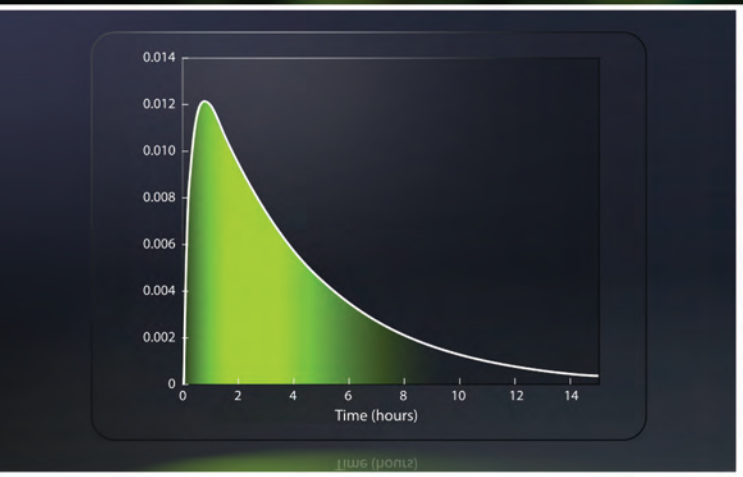

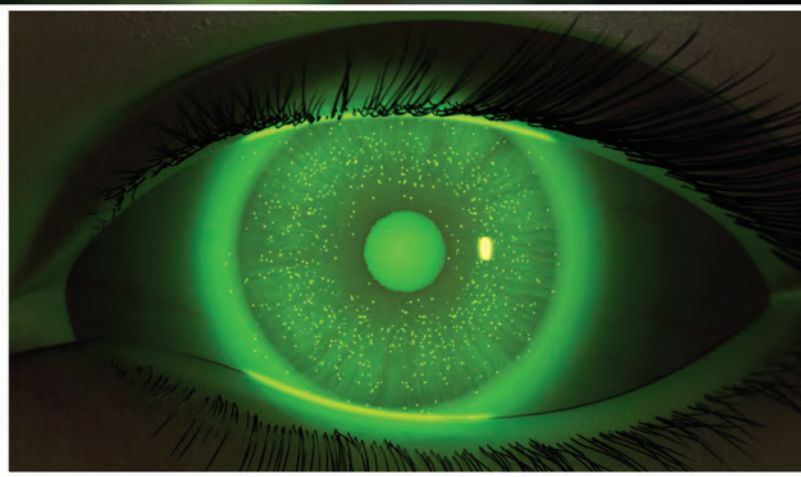

The Science Behind the "Stain"

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The Science Behind the "Stain"

Paul Karpecki, OD, FAAO, graduated from Indiana University and completed a fellowship in Cornea and Refractive Surgery at Hunkeler Eye Centers in affiliation with the Pennsylvania College of Optometry in 1994. He currently works at the Koffler Vision Group in Cornea services and Ocular Disease Research. Dr. Karpecki is the current Chair of the Refractive Surgery Advisory Board to the AOA, Chair of the ASCRS Task Force on the Integrated Eyecare Delivery System and serves on the AOA Congress Education Committee. A noted educator and author, Dr. Karpecki presently serves on eight professional journal editorial boards.

Frank Bright, BS, PhD, is the A. Conger Goodyear Chair in Chemistry and State University of New York & University of Buffalo Distinguished Professor of Chemistry and an expert in fluorescence spectroscopy. Professor Bright has co-authored more than 300 peer reviewed journal publications, delivered over 800 conference and university lectures, and holds 11 issued U.S. patents. He has served on numerous advisory and journal editorial boards. He has been awarded the 3M Non-Tenured Faculty Award (1988-'91), the Eastern New York American Chemical Society Buck-Whitney Medal (1999), the SUNY Chancellor's Award for Excellence in Teaching (2000), the New York Section of the Society for Applied Spectroscopy Gold Medal (2003), the Akron Section Award of the American Chemical Society (2003), the A.A. Benedetti-Pichler Award in Microchemistry from the American Microchemical Society (2005), the Jacob F. Schoellkopf Medal of the Western New York American Chemical Society (2006), and was elected a Society for Applied Spectroscopy Fellow (2010).

Nathan Efron, BScOptom, PhD, DSc, FAAO(Dip CCLRT), FCCLSA, FBCLA, FIACLE, FACO, is Research Professor in the Institute of Health and Biomedical Innovation at the Queensland University of Technology, and holds a joint appointment in the School of Optometry. He has served as president of both the Contact Lens Society of Australia (1981) and the British Contact Lens Association (1997). He lectures extensively worldwide, particularly in the field of the ocular response to contact lens wear, and has published over 700 scientific papers, abstracts and textbook chapters, and has written/edited six books—his most recent is "Contact Lens Practice," 2nd Edition (Butterworth-Heinemann, 2010). He has won numerous research awards, including the BCLA Gold Medal (2001) and American Academy of Optometry's Glenn Fry Award (2010). Professor Efron has a special interest in the clinical recording of corneal staining and has developed a validated pictorial grading system for this purpose which has been distributed worldwide.

Philip Morgan, BSc, PhD, MCOptom, FAAO, is director of Eurolens Research at The University of Manchester where he is a senior lecturer and program director for optometry. Dr. Morgan is a vice president of the International Society for Contact Lens Research and secretary of the International Association of Contact Lens Educators. He is an honorary member of the Association of Contact Lens Manufacturers, a Fellow of the American Academy of Optometry and the British Contact Lens Association and a member of the College of Optometrists. He has authored over 160 papers, primarily relating to contact lens clinical performance and the nature of international contact lens markets. Dr. Morgan is currently undertaking research into mechanisms of corneal staining and has recently authored a review article on this important topic.

Moderator: *Paul Karpecki, OD, FAAO*

It has been almost five years since I first published the article entitled "Much Ado About Staining" in *Review of Optometry*, which explored what we really knew in 2006 about the relationship between "corneal staining" and contact lens multipurpose solutions (MPS). This was published just prior to the controversial "staining grid." While the Grid showed MPS-associated hyperfluorescence under the slit-lamp at two hours, it did not explain the "what" or "why" behind it; even so, many proponents of the Grid continue to suggest that it shows us which solution/lens combinations are "biocompatible" and which are not. New evidence suggests that the preservative-associated transient hyperfluorescence (or PATH) observed at two hours after lens insertion is a benign phenomenon due to an interaction between fluorescein, MPS preservatives, and corneal cell membranes. The misinterpretation of PATH as "real" corneal staining, like that observed in pathological conditions, may be due in part to the fact that there is not a lot of teaching regarding the true properties of fluorescein and what is actually occurring when we see either PATH or corneal staining.

To discuss the science of fluorescein, corneal staining, and PATH, I have asked some of the preeminent research experts in the study of fluorescence spectroscopy and corneal staining from around the world to share their new research and personal opinions on these topics.

Section 1: Understanding Fluorescein

Dr. Karpecki: Dr. Bright, as this is your field of study, can you share some of your knowledge about fluorescein and its properties with us?

Dr. Bright: My first publication in graduate school was on fluorescein. You might say I am where I am because of, or in spite of, my initial experience with fluorescein.

Fluorescein is a molecule not found in nature. Fluorescein, originally synthesized in 1871 by von Bayer, is a fluorescent molecule commonly used for geological tracer studies, in forensic science and as a label in cellular and molecular biology.[1-4] In the optometry and ophthalmology literature, one can trace clinical fluorescein use back to the 1880s.[5]

If you are willing to indulge the chemist in me, I'll share some of the finer points about fluorescein. In aqueous media, fluorescein exhibits well-understood hydrolytic equilibria (pK_1 = 2.08, pK_2 = 4.31, and pK_3 = 6.43) and it can exist in cationic (one positive charge), neutral, anionic (one negative charge), and dianionic (two negative charges) forms depending on the solution pH.[6] Both negatively charged states of fluorescein emit fluorescence, though the dianionic state is the more luminous of the two. Under the pH conditions encountered within the human pre-corneal tear film (pH = 7.8 ± 0.1), the most prevalent fluorescent form of fluorescein is the dianion (FL^{2-}), at approximately 80% dianion to 20% anion.[7] Fluorescein absorbs light between 450 and 500 nm and fluoresces between 500 and 600 nm.[8,9]

Traditionally, we are taught that the fluorescence intensity is directly proportional to the concentration of the fluorescing species. This turns out to be a major oversimplification that is highlighted by, as it turns out, fluorescein. Specifically, how luminescent fluorescein is depends strongly on its local concentration and on the local pH. By "local" I mean the immediate environment surrounding the fluorescein molecule. As the pH surrounding fluorescein decreases, there is a dramatic decrease in the amount of luminescence observed because the anionic form gives off less fluorescence than the dianionic form.[6,7,9] Thus, if you have equal numbers of fluorescein molecules reporting from environments at pH 7.5 and 6.0, the fluorescence you will see from the pH 6.0 range (10% dianions/ 90% anions) will be significantly less bright versus the fluorescence from the pH 7.5 range (75% dianions/ 25% anions).

Because fluorescein exhibits a small difference between its absorbance and emission spectrum, they overlap to a significant degree, thus, if the local fluorescein concentration becomes too high, the fluorescein emission can undergo self-quenching and the emission actually decreases as the fluorescein concentration increases. In an extreme case, one measures low fluorescence intensity and the local concentration of fluorescent species is actually quite high as is evidenced by the fluorescence actually increasing as the sample is diluted to a point where the luminescence then decreases linearly as the sample concentration is further diluted. This is the exact opposite of what intuition tells you and it can lead to obvious misinterpretation of experimental data.

Dr. Karpecki: How specific is fluorescein? Is it a "molecular probe," that is, a group of molecules that attaches to another molecule or cellular structure used to study their properties?

Dr. Bright: This is a fascinating question, and it points to the misguided view of fluorescein being a molecular probe. In most molecular targeting experiments, a molecular probe is one or more fluorescein reporter molecules covalently (permanently) attached to a molecule, such as an antibody, enzyme, DNA, or RNA, that targets a specific cellular or extracellular component, not the fluorescein.[10-13] Although fluorescein is widely used in clinical practice and research to evaluate the ocular surface, some individuals try to infer molecular-level changes of cellular components. I find this extremely surprising given that fluorescein by itself is not a bona-fide molecular probe. In the fluorescence spectroscopy community where I come from, fluorescein is a label (a molecule that allows for detection/ localization of the probe to which it is attached), not a probe.[14] This fact is one reason I was drawn to the desire of many in the optometric community to determine the hard science behind the MPS-associated "corneal staining" phenomena.

Dr. Karpecki: Dr. Efron and Dr. Morgan, do you feel that the optometric community is aware of the properties of fluorescein and how it affects their observations?

Dr. Morgan: The use of fluorescein to demonstrate apparent abnormality at the ocular surface is very long-standing and its use pre-dates all living eye care practitioners. Perhaps because of its history, little modern day attention is given to its physiochemical properties and the underlying mechanisms behind its interaction with the tissues at the ocular surface. In fact, surprisingly little fundamental research is available in the literature, and I think it is fair to say that we tend to rely on assumption, intuition and extrapolation when considering the clinical impact of fluorescein staining rather than basing decisions on hard evidence.

Dr. Efron: Contact lens practitioners have long been using fluorescein to assess the fitting of rigid lenses in addition to assessing ocular health. In fact, the first recorded use of fluorescein in contact lens practice can be traced back to the early 1960s. However, as mentioned by Dr. Morgan, despite

being very familiar with this staining compound, little attention has been paid to understanding its physicochemical properties, and how this translates to an interpretation of what is actually happening when we see "fluorescein staining." In the modern age, the cutting edge of developments in biomedical science occurs at the interface of different disciplines. In this regard, I believe we are fortunate to have the input from Dr. Bright—a renowned chemist who has vast expertise in the chemical properties and behavior of fluorescent molecules. Translating his understanding and knowledge in this area will undoubtedly assist ophthalmic clinicians in gaining a better appreciation of what "fluorescein staining" really represents.

Dr. Karpecki: From my experience, I agree that eye care practitioners, including myself, were not aware of fluorescein's properties and how these properties affect what is seen under the slit-lamp. I think, because it is a quick and easy test that can be done in almost any office, eyecare practitioners (ECPs) simply look at what they are observing rather than seeking to understand what it actually represents. Working with people who have dry eye, you learn that it is essential to understand how a test works and what affects the results and why, in order to properly diagnose these patients since many test results are often counterintuitive and fluorescein testing is one of them. For instance, it is not uncommon for one of my dry eye patients to present with complaints of significant symptoms such as burning, grittiness or scratchiness, without corneal staining or other clinical signs such as conjunctival hyperemia, reduced tear break-up time, or conjunctival staining. At the other end of the spectrum, some patients may claim that they're virtually symptom-free, yet, when you look under the slit-lamp, they light up like a Christmas tree with significant corneal and conjunctival staining.

Dr. Karpecki: Based on fluorescein's properties, do you feel it is a reliable diagnostic test?

Dr. Morgan: I believe that in many situations, fluorescein staining does indicate the presence of some sort of 'defect' at the ocular surface, such as that observed in foreign body staining, where one can actually see the trajectory the foreign object traveled across the cornea and caused mechanical damage to the corneal epithelium. However, precisely what is being 'stained' is not currently clear, since it is unknown what fluorescein actually binds to within the cornea or in corneal cells and may differ based on the cause of the hyperfluorescence.[15] In some clinical situations, this may be of no importance in that appropriate decisions can be made without a full understanding of the relationship between fluorescein and the ocular surface. At other times—with "solution-induced staining" perhaps—a better understanding as to the underlying mechanisms would be useful to aid in optimum patient management. This is why research is ongoing to delineate the etiology of the increased hyperfluorescence observed in users of MPS.

Dr. Efron: Fluorescein has some limitations, as will be discussed in this forum, but it has the advantage of being inexpensive, readily available and physiologically inert (i.e., non-toxic to the eye) in the majority of individuals. It is capable of revealing disruptions to corneal integrity. However, high levels of hyperfluorescence can occur at certain time points when used in contact lens wearers using certain preservative-based solutions, which can result in a bright signal that may be misinterpreted as corneal compromise. So, used properly, and with a good understanding of fluorescein's physicochemical properties, fluorescein will continue to have an important role in ophthalmic practice. For example, complications such as superior epithelial arcuate lesions or SEALs can only be diagnosed based on their characteristic corneal staining pattern.

Dr. Bright: I think the utility of fluorescein is clear given the fact that people in very disparate disciplines also use the exact same "fluorescein" for doing things far different than the assessment of corneal health. I think that the possibility for misinterpreting the fluorescence from a non-molecular, albeit strongly and conveniently emitting fluorescent molecule like fluorescein, is something the ophthalmic community should consider very deeply.

Dr. Karpecki: Because of the limitations of fluorescein staining, I do not use it alone in diagnosis. When I do perform ocular surface staining, I always use it in combination with lissamine green or after osmolarity testing for example. With the addition of lissamine green, I can assess the entire surface of the eye and not only the cornea. The results are analyzed within the context of the other findings from the patient's history and exam. Most often, the use of fluorescein dye by itself does not give enough information for an accurate diagnosis or gives results that can be misinterpreted.

Section 2: Fluorescein and Preservative Interactions: Cause and Effect

Dr. Karpecki: Dr. Bright, can you tell us about your ongoing research into the molecular basis of PATH?

Dr. Bright: Uptake of MPS preservative agents, such as polyhexamethylene biguanide hydrochloride (PHMB), polyquaternium-1 (PQ-1), alexidine, and myristamidopropyl dimethylamine (MAPD) known by the trademark name Aldox (Alcon, Inc.), by the contact lenses has been hypothesized as the root cause of transient corneal hyperfluorescence associated with MPS.[16]

With this as our starting point we

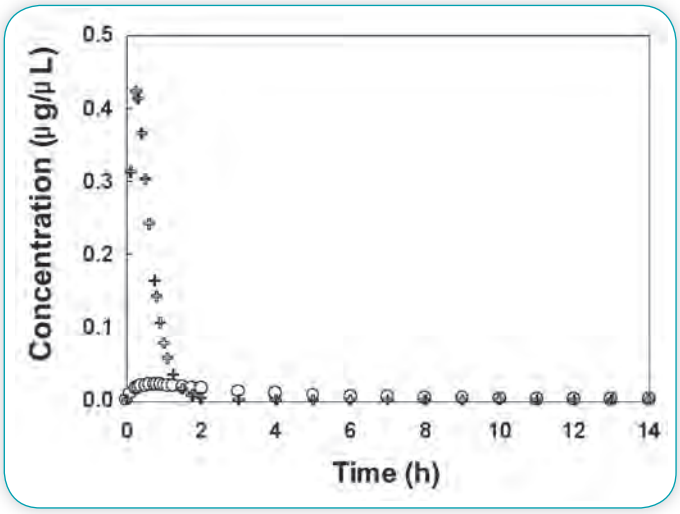

Figure 1. Aldox (+) and PHMB (0) concentration at the corneal epithelial cell surface based on release data from Powell et al (balafilcon A contact lens [CL]) over time.[16] Volume between CL and corneal surface = 3 µL. Tear film clearing rate = 11-13 µL/h.

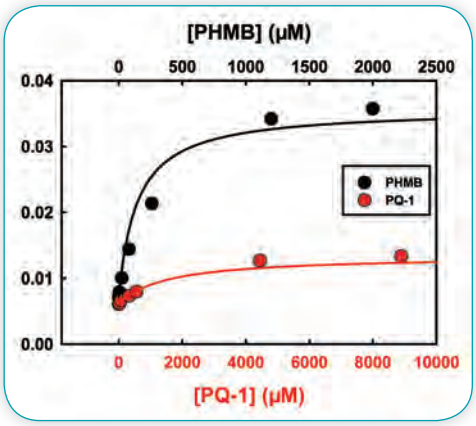

Figure 2. Binding equilibrium curves (37 °C) for fluorescein (FL) with PHMB and PQ-1 at pH 7.5. Both preservative agents bind FL; PHMB binds to FL an order-of-magnitude more strongly in comparison to PQ-1.

began our research by asking some simple questions including:

- *What is the extent and time course of preservative agent uptake by and release from contact lenses?*
- *What are the basic properties of fluorescein (FL) and commonly used MPS preservative agents, such as PHMB and PQ-1?*
- *Is there an association between FL and PHMB and/or PQ-1?*
- *Do FL, PHMB, and/or PQ-1 associate with the corneal epithelial cell surface? And if so, what is the impact?*
- *If FL doesn't associate with corneal epithelium components, do PHMB and/or PQ-1 mediate FL association with the corneal epithelium?*

Using data from Powell et al. on PHMB and Aldox release from a balafilcon A contact lens, we plotted the release rate at each time point on the same graph (rather than the cumulative concentrations graphed in the paper).[16] The results shown in Figure 1 have several interesting features. First, preservatives are taken up and released by contact lenses. Second, uptake and release are based on the specific properties of the contact lens material and the preservative's properties. Third, the peak Aldox concentration released from the lens is approximately 20-fold greater compared with PHMB peak concentration release from lenses. Finally, the Aldox concentration peak occurs within 30 minutes, while the PHMB concentration peaks within two hours. If the hypothesis is correct and preservative uptake and release by lenses is the basis for the transient hyperfluorescence phenomenon, which I believe it is, then these results point directly to a possible artifact of using a single time point measurement, such as two hours post-lens insertion for assessment. You run the risk of missing something if you only look earlier or later. The problems of single time point measurements are well known in my community and for this very reason, we have the entire fields of kinetics and time-resolved measurements.

Earlier, I mentioned that fluorescein is in large measure a dianion (i.e., FL^{2-}) at the ocular surface pH. Interestingly, PHMB and PQ-1 exist as cations under the same pH conditions.[17-19] It is well known that molecules of opposite charge tend to be attracted to one another. So the positively charged PHMB and PQ-1 may associate with negatively charged FL.

To determine if FL associates with PHMB or PQ-1, we used a technique called fluorescence anisotropy that is commonly used in my field to determine whether there is an association between molecules and quantify the strength of the association.[20, 21] For those who may not be familiar with fluorescence anisotropy, it measures the rate at which molecules rotate or "tumble" as they are constantly moving. The smaller a molecule is, the faster it rotates. Molecules rotating more slowly show higher fluorescence anisotropy and smaller more quickly rotating objects show lower fluorescence anisotropy. When two molecules interact, they act as one larger unit rather than two separate units, so the anisotropy goes up. Since these are equilibrium reactions, the stronger the association, the greater the number of molecules interacting at one time leading to higher levels of fluorescence anisotropy.

Figure 2 presents the binding curves of FL to PHMB and PQ-1 at body temperature (37 °C). At the pH and osmolarity similar to the human tear film, free FL clearly interacts with both preservatives. However, FL's association is at least an order-of-magnitude stronger to PHMB in comparison to PQ-1 (p <0.0001). Also, though not

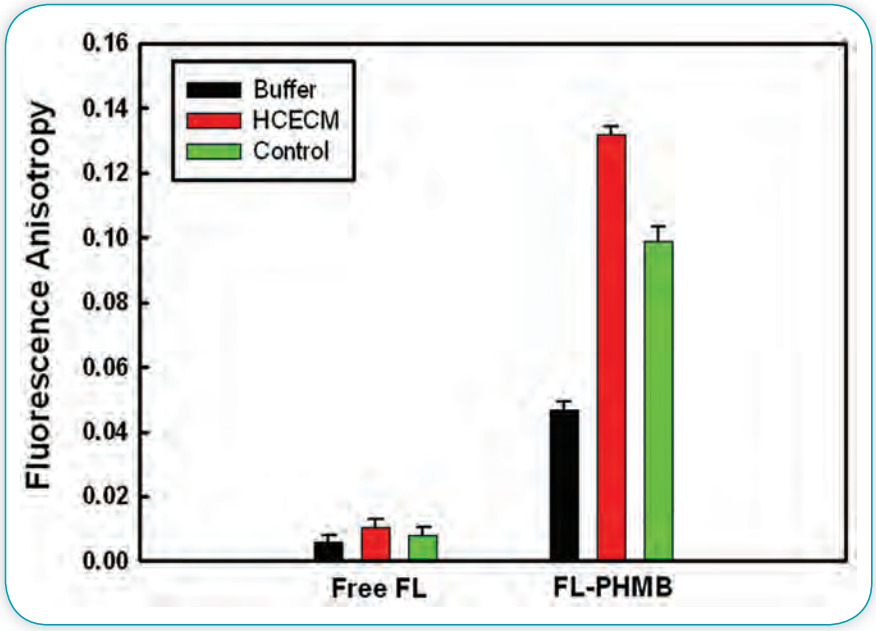

Figure 3. Interactions of free fluorescein dianions (FL) and fluorescein (dianion)-labeled PHMB (polycation) (FL-PHMB) associating with Control and HCECM liposomes. Results show PHMB associates with the liposome's surfaces; free FL does not associate with either liposome.

shown, FL's attraction for PHMB can be up to 50x stronger in comparison to PQ-1.

We were also interested in whether fluorescein, PHMB, and/or PQ-1 associate with corneal cell membranes and further to understand if they disrupt these corneal cell membranes. To determine this, we used liposomes, which are well-established tools that have been used for decades to study a wide range of complex biological issues with molecular-level detail and insight.[21-27] Since the composition of the corneal cell membrane is very complex, we needed to simplify the system if we were going to determine what fluorescein, PHMB, and/or PQ-1 is binding to, if they bind at all. Since previous researchers have already shown that PHMB can bind to certain phospholipids, using liposomes was a good starting point. Fortunately for us, the phospholipid composition of the human corneal epithelium has already been determined.[24, 28] This allowed us to create a relevant liposomal model composed of the major components of the human cornea to accurately assess the cellular- and molecular-level interactions of preservatives with the superficial surface of the corneal epithelial cell membrane.

Figure 3 presents the interaction (or lack therof) of free FL and fluorescein-labeled PHMB (FL-PHMB) in the presence of two liposome types, one a Control (C) and the other a human corneal epithelial cell mimic (HCECM). As I previously stated, FL is smaller than a FL-PHMB complex, and therefore has a lower level of fluorescence anisotropy as seen by the difference in the black bars. The data shows two key points. First, free FL does not associate with either liposome. Second, FL-PHMB associates with both liposome types ($p < 0.001$).

To assess the impact of PHMB and perhaps PQ-1 association with the corneal epithelial surface we again turned to our liposome models and fluorescence anisotropy measurements. In this experiment, we used fluorescence anisotropy measurements again to determine the effect of PHMB and PQ-1 associating with the liposome by measuring the stability of the liposome using its

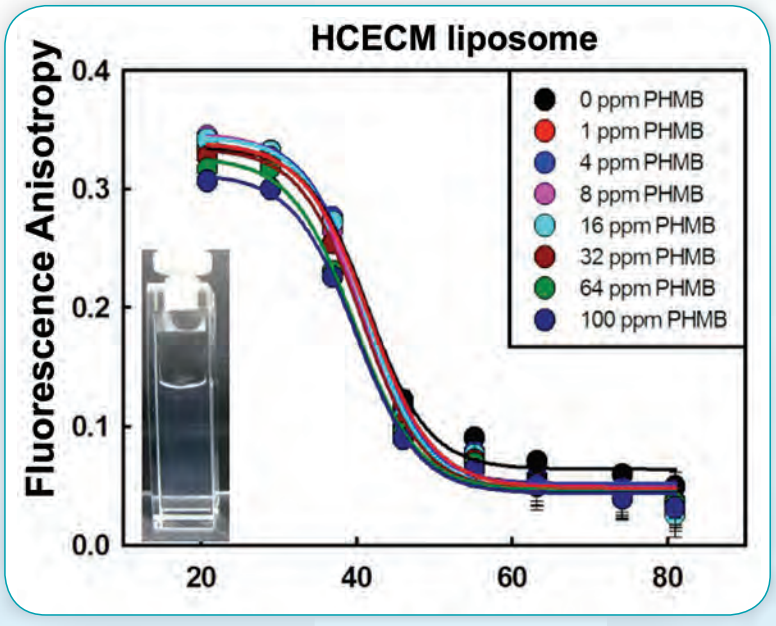

Figure 4. PHMB-dependent (0-100 ppm) steady-state fluorescence anisotropy vs temperature profiles. A photograph of the liposome solution in the presence of 100 ppm PHMB is also shown; no precipitant is observed.

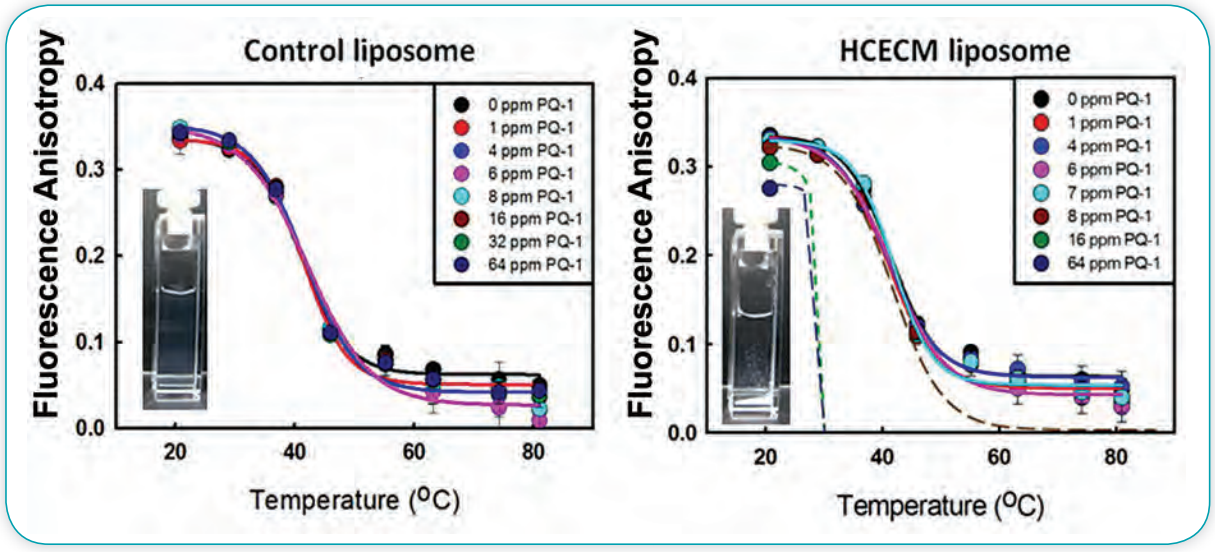

Figure 5. Effect of PQ-1 on control liposome integrity with a photograph of a standard cuvette containing 64 ppm PQ-1 (left panel) and on HCECM liposomes with a photograph of the liposome solution in the presence of 8 ppm PQ-1 (right panel); a white precipitate is clearly visible in the bottom of the cuvette. Hatched lines (8, 16 & 64 ppm) are hypothetical stability curves as liposomes fell out of solution.

melting temperature (T_m). Liposome T_m measurements have been used for many decades as a tool to assess membrane integrity amongst other phenomena.[21-27]

Figures 4 and 5 show the effects of PHMB's and PQ-1's association, respectively, on the membrane integrity of HCECM and Control liposomes. So, a significant increase in T_m means that you need to increase the temperature to disrupt the liposome; therefore the liposome becomes stable. Similarly, if T_m is reduced, it means the liposome becomes less stable.[21-27] Inspection of Figure 4 shows that at physiological temperature (37 °C), HCECM liposomes are unaffected by the presence of up to 100 parts per million (ppm) or 0.01% PHMB. While not in the figure, the same result is observed with Control liposomes up to 100 ppm PHMB. The results in Figures 3 and 4 together demonstrate that PHMB binds to the HCECM membranes that contain the major, key components of the human corneal epithelium and does not have a deleterious impact on the HCECM membrane integrity at up to 100 ppm PHMB, which is up to 100-times the PHMB levels found in commonly used MPS.

As shown in Figure 5, the situation with PQ-1 is very different in comparison to PHMB. As observed with PHMB, the Control membrane T_m is not affected by a high concentration of PQ-1 of up to 64 ppm PQ-1. So, this association with the Control membrane does not affect the Control's membrane stability at up to 64 ppm PQ-1, which can be seen by the clear fluid in the cuvette in the left panel. Also, the HCECM membrane stability (T_m) is not affected by up to 7 ppm PQ-1 as shown in the right panel; however, above 7 ppm PQ-1, the HCECM membranes are completely and irreversibly damaged and the liposomes fall out of solution. Precipitation in the cuvette is only seen for the HCECM membranes with ≥8 ppm PQ-1. This result demonstrates the damaging effects PQ-1 at high concentrations can have on the HCECM membranes.

Interestingly, some commercial MPS products contain up to 10 ppm PQ-1. Additional research is currently underway to further understand this phenomenon and the potential implications of these findings.

Dr. Karpecki: Thank you for taking the time to walk us through these important findings. It was over a year ago that I was first introduced to some of this work and it helped me understand why we were seeing this transient hyperfluorescence with fluorescein in MPS users who were free of any additional signs or symptoms we would expect to see with the "staining" observed and unlike pathological staining, resolved without treatment.

Dr. Karpecki: I would love to hear the thoughts of the other participants regarding Dr. Bright's findings?

Dr. Efron: Dr. Bright's findings have potentially important implications for contact lens practitioners. The first, and perhaps most obvious conclusion, is that practitioners should anticipate observing a strong hyperfluorescent signal— which we now refer to as PATH—after about two hours in patients who have been wearing soft lenses preserved in a PHMB disinfecting solution, and should not be alarmed by it! Additionally, there is some evidence that indicates PATH is observed earlier with PQ-1/Aldox-based MPS, so clinicians should be

aware that patients using solutions with these preservatives may observe PATH before two hours.[16,29,30] Of course, practitioners should remain alert as to the possibility of the presence of any genuine coincidental pathology. One possible way of distinguishing between PATH and pathological corneal staining would be to have the patient return in, say, another four hours. By this time, the PATH signal would have largely subsided, so substantial residual hypofluorescence may be corneal staining and indicative of some disturbance to the ocular surface, especially if there are other concomitant signs or symptoms.

Dr. Morgan: Dr. Bright's findings suggest that alternative mechanisms appear to exist to explain some forms of fluorescein staining such as what some call PATH. I believe that we should use his findings to challenge our perceptions of fluorescein staining and encourage further research to deliver knowledge in this area to eye care practitioners.

Dr. Karpecki: Dr. Bright, does fluorescein bind to all preservatives in MPS or only PHMB and PQ-1?

Dr. Bright: So far we have only completed experiments with PHMB and PQ-1. We are, however, in the midst of experiments using other preservative agents. But based on the physiochemical properties of alexidine and Aldox, which both carry an overall net positive charge, we expect that both preservatives will interact with fluorescein, though what is the extent and strength of their binding is the real question.

Dr. Karpecki: Are these associations between fluorescein and preservatives reversible?

Dr. Bright: As the associations formed between fluorescein and preservatives are equilibrium complexes, rather than permanent associations, such as covalent bonds, the association we are discussing here is completely reversible. Thus, the degree to which the complex forms (percentage of molecules of a given type that are actually in a complex and not free in solution) depends on, amongst other things: (i) temperature, (ii) pH, (iii) instantaneous concentration of the molecules in play, including PHMB, PQ-1, fluorescein, other agents within the MPS that can associate with FL and/or the preservative agents [Figure 1]), and (iv) secondary additives and/or factors, such as rinsing solutions, blinking, and eye drops with or without additives that could themselves associate with one or more of the players. For example, adding additives like citrate buffers is known to bind up the preservative agents, which has helped reduce the hyperfluorescence phenomenon even with high concentration PQ-1 solutions.[31] Based on our studies, the magnitude of the association that we have measured for PHMB and PQ-1 with FL are several orders-of-magnitude smaller in comparison to a typical antigen-antibody association. Thus, if we dilute our solutions, the complexes dissociate soon after dilution based upon the strength of the ionic interaction; antigen-antibody complexes do not dissociate on a similar time scale, if at all.

Section 3: Corneal Staining and MPS-Associated Hyperfluorescence: Is it all the same?

Dr. Karpecki: Dr. Morgan, I know that you have been actively exploring mechanisms of fluorescein staining at the cellular level both with and without MPS. Could you please tell us a little bit about that work?

Dr. Morgan: We have developed some cell culture models to investigate corneal staining and our findings suggest that most cells will uptake fluorescein and appear dark green microscopically (fluorescent cells), with a minority taking up much greater amounts, appearing bright green (hyperfluorescent cells). We have modelled staining, which is related to contact lens care systems and in doing so, we found many more bright green cells when compared to exposure to saline, mimicking the clinical situation. A key outcome is to understand why these cells appear bright green. Our work continues in this area, but at this stage it does not seem that these cells are dead. In fact, we believe these bright green cells are normal and active. Our early findings agree with those from a previous study by Wilson and colleagues that was published in 1995.[32] In their study, they observed in untreated healthy corneas a typical micropunctate pattern of corneal staining due to hyperfluorescent epithelial cells that took up fluorescein. In addition to hyperfluorescent cells, many other cells stained with fluorescein, although less brightly. They hypothesized that hyperfluorescent cells were those that took up fluorescein optimally.[32] This may be occurring in our model system as well.

Dr. Karpecki: Do we know what fluorescein actually stains in the cornea?

Dr. Morgan: It seems quite possible that similar appearances of ocular surface hyperfluorescence can have significantly different causes. For example, we generally describe the pooling of fluorescein in the micro-depressions formed when air bubbles are trapped under a rigid contact lens as 'dimple-veil staining'. Similarly, the depressions formed by mucus balls under a silicone hydrogel lens can fill with fluorescein and the term 'staining' is often applied. It seems evident that these phenomena are not "staining" in the cell biology sense of the term. On the other hand, it seems possible that other appearances of ocular surface hyperfluorescence are indeed

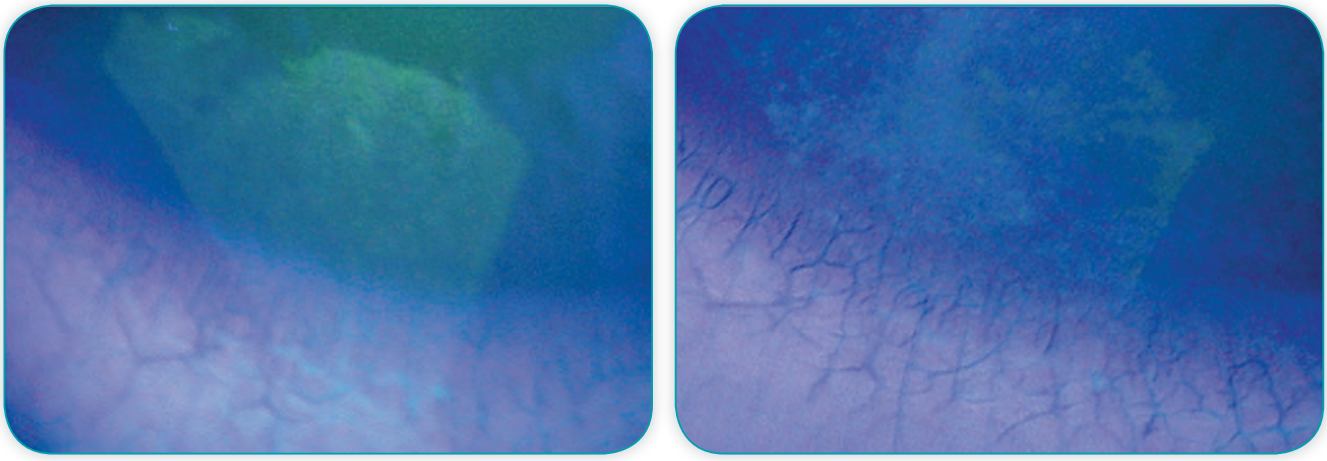

Figure 6. Photograph of the fluorescein-stained cornea in healthy subject immediately post impression cytology (left panel). Photograph of the fluorescein-stained impression defect three hours later (right panel). Reproduced with permission from Thinda S, Sikh PK, Hopp LM, et al. Polycarbonate membrane impression cytology: evidence for fluorescein staining in normal and dry eye corneas. *Br J Ophthalmol* 2010;94:406-409.

"staining"—with fluorescein entering the cells. Solution-induced 'staining' might be due to one of these processes or could be some other phenomena altogether, including MPS preservative and fluorescein interactions shown by Dr. Bright's group. There is an obvious requirement for better knowledge across this entire area.

Dr. Efron: Prior to 2010, two preclinical studies showed that fluorescein can enter healthy cells but the level of fluorescence could not be seen under a biomicroscope with a blue filter.[32,33] Recently, two clinical studies published in the ophthalmology literature by researchers at the Jules Stein Eye Institute at UCLA using membrane cytology and confocal microscopy showed that healthy human corneal epithelial cells in vivo do indeed take up fluorescein.[34, 35] The first study demonstrated that removing the mucin layer in an individual in good ocular health led to "fluorescein" staining. As you can see in the left panel of Figure 6, the "staining" pattern matches the size and shape of the membrane used for cytology. When they inspected the membrane used for the cytology, the number of epithelial cells they had removed could not account for the hyperfluorescent pattern and the membrane stained positively for mucin. As you can see in the right panel of Figure 6, after three hours, the "staining" had all but resolved.[35]

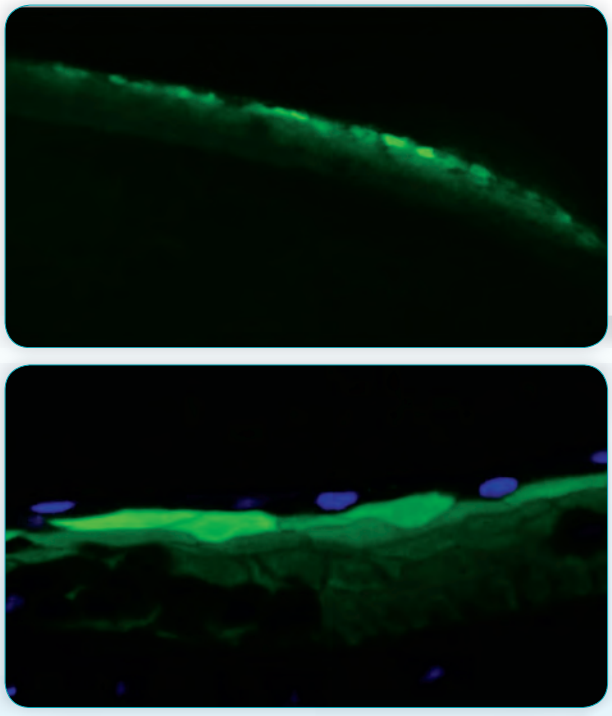

Figure 7. Fluorescent photograph of the corneal button demonstrates pre-operative fluorescein is still present in the tissue of the cornea in cross-section (Top). After DAPI staining of the cell nuclei, punctate stains appear in the second cell layer (bottom). Reproduced with permission from Mokhtarzadeh M, Casey R, Glasgow BJ. Fluorescein Punctate Staining Traced to Superficial Corneal Epithelial Cells by Impression Cytology and Confocal Microscopy. *Invest Ophthalmol Vis Sci.* 2011;52(5):2127-35.[34]

Similarly, in the second study, removal of the cornea post-fluorescein dye testing showed that fluorescein enters the cells of the epithelial layer (Figure 7, top panel).[34] In the bottom panel of Figure 7, they found that fluorescein enters cells throughout the superficial epithelium, so these cells are not desquamating. While these studies did not look at the hyperfluorescence observed in users of MPS, they certainly cast great doubt as to what was thought to be the mechanisms of "corneal staining." These findings in conjunction with Dr. Morgan's show that even if fluorescein does fill intracellular spaces or enters dead or damaged cells, which we can't be sure of since fluorescein is not a molecular probe; these are not the mechanisms at play here.

Dr. Karpecki: Are all preserva-

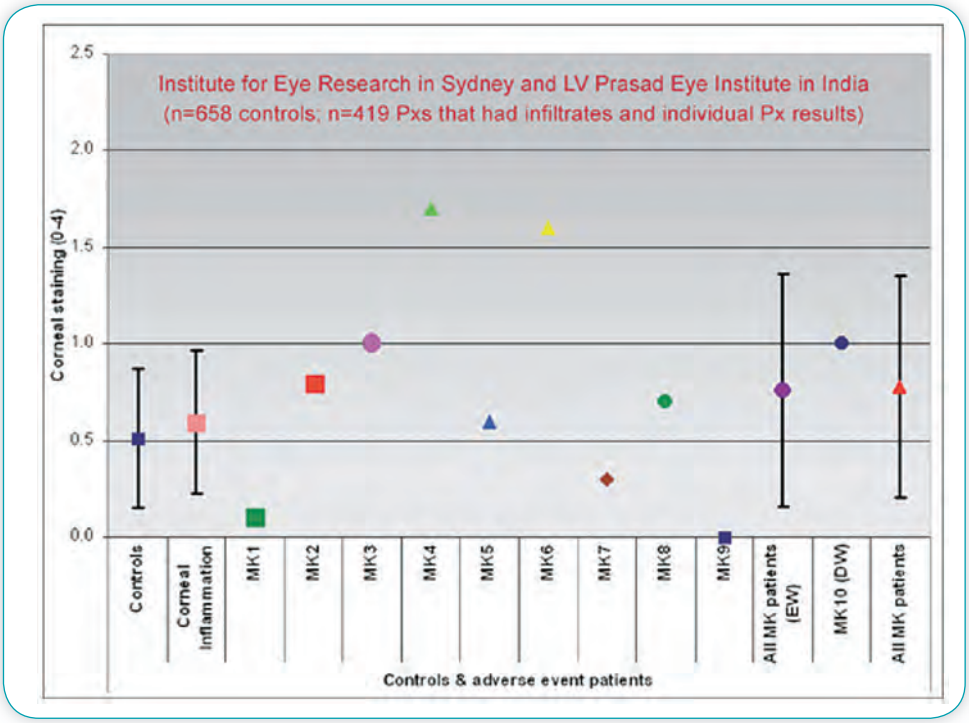

Figure 8. No relationship observed between the extent of corneal staining and microbial keratitis (MK). Average corneal staining (or PATH) extent prior to an MK event among 10 individuals shown. There is no pattern or trend of the extent of staining and these 10 events. In the control group (n=658) and the group who had corneal inflammatory events (n=419), no significant difference was found in corneal staining extent when compared. Reproduced with permission from Carnt N, Willcox MD, Evans V, et al. Corneal staining: The IER Matrix Study. *Contact Lens Spectrum* 2007;22(9):38,40-41,43.

tives adsorbed and released by contact lenses?

Dr. Bright: Based on my studies as well as those published in the literature, all preservatives are absorbed and released by all soft contact lenses.[16]

The rate of which, depends on the specific lens material and preservative.[16]

Dr. Karpecki: How do you tell the difference between PATH and pathological corneal staining?

Dr. Efron: PATH is generally observed with peak hyperfluorescence occurring two hours following insertion of a lens that has been stored in PHMB-based MPS, or earlier for PQ-1–based MPS. As I stated earlier, one possible way of distinguishing between PATH and pathological corneal staining would be to have the patient leave their contact lenses out and return in a few hours. By this time, the PATH signal would have largely subsided, and any residual corneal staining might be indicative of some disturbance to the ocular surface. PATH is generally even and perhaps more prominent in the mid-peripheral cornea, coinciding with slightly greater bearing of the soft lens against the cornea in those regions. As well, PATH is usually bilateral, so significant unilateral staining may indicate corneal pathology.

Dr. Karpecki: I also look at timing since it can be expected that PATH will be observed for several hours after lens insertion. So observing the patient later in the day, without treatment or removal of the contact lens, will typically show no staining. Similar to what Dr. Efron mentioned, PATH has an annular appearance, where corneal staining during asymptomatic contact lens wear shows characteristic patterns, such as 3 and 9 o'clock staining in rigid lens wearers and SEALs in silicone hydrogel wearers. Further, with the exception of the two examples I just gave, signs such as conjunctival injection, and symptoms, such as irritation, burning, or photophobia, are often present with pathological corneal staining, which helps to differentiate PATH from contact lens complications.

Dr. Karpecki: Does PATH signify a breakdown in the corneal epithelial barrier?

Dr. Efron: No, PATH appears to be a strong signal resulting from a chemical interaction with contact lens solution preservative agents. It is probably not indicative of any form of pathology or breakdown of the corneal epithelial barrier.

Dr. Morgan: At this time, it is difficult to provide a definitive answer to this question for all cases since we have only begun to explore the cause of PATH at a cellular and molecular level. Then again, in general, corneal staining which is associated with solution use is transient and superficial. Most reports suggest that it is asymptomatic and there is no real evidence to link it to an increase in contact lens-associated infections. This might suggest that there is no breakdown of the corneal epithelial barrier.

Dr. Karpecki: If PATH did signify a breakdown in the corneal barrier, then infection would be a sequelae of PATH, but studies, such as that by clinical researchers at the Brien Holden Vision Institute in Australia (Figure 8), show that observed hyperfluorescence is not predictive of future infection.[36] To date, I have not seen any evidence in the literature or the clinic that supports PATH increasing the risk for infection, which would be expected if the cornea was breached.

Section 4: Applying New Findings to Clinical Practice

Dr. Karpecki: We have previously discussed Dr. Bright's findings regarding the benign interactions of the MPS preservative PHMB, with fluorescein and the corneal cell membrane as well as, in Dr. Morgan's research, the ability of fluorescein to enter healthy cells and even show hyperfluorescence in some cells in the absence or presence of PHMB-based MPS with no harmful effects. I am interested in how these new understandings can help guide clinical decision making.

Dr. Karpecki: In light of all the new findings, how valuable do you feel the Staining Grid is in informing clinical decisions?

Dr. Efron: Paradoxically, the Staining Grid can be useful in informing clinicians about what to expect to observe in contact lens wearers when fluorescein is used at a two hour time point following lens insertion from certain MPS solutions, as long as it is understood that this grid is really only presenting information about PATH at two hours, rather than about corneal dysfunction or solution and lens biocompatibility. If this is properly understood, then the Grid can be useful in alerting practitioners of the strength of the PATH signal with certain lens-solution combinations. However, the grid may not be useful if you examine contact lens patients at other timepoints, such as 30 minutes after lens insertion, as you may see higher levels of PATH with PQ-1/Aldox-based solution than would be expected based on the Grid. This is one of the significant limitations of the grid for clinical practice. Practitioners can then ignore a PATH signal and concentrate on looking for pathological corneal staining. It is important to understand that the PATH signal is benign, and should not form the basis of clinical decision-making.

Dr. Morgan: The Staining Grid presents reasonable figures for the peak level of staining with certain lens/solution combinations at just one point in time—two hours. To this end, eye care practitioners examining patients after about two hours of lens wear might reasonably expect to see the designated area of corneal staining and this might be of reassurance that a patient is showing a "normal" response similar to others wearing the same lenses and using the same solution.

Dr. Karpecki: Since its inception, I have not found the Grid to be informative as to which patients are likely to have complications based on the lens and solution they are using. I believe that a staining grid within 30 minutes of lens insertion would be more helpful since we know that if pathological staining were to occur it would be imminent after contact with a solution or lens (e.g., a patient accidentally putting a non-neutralized, hydrogen peroxide immersed contact lens in the eye). Though, as previously stated, the Grid can inform clinicians as to what amount of PATH they can expect to see within two hours after lens insertion even though it may not be related to any sequelae or pathology.

Dr. Karpecki: I have also come to expect at least some low grade normal physiological corneal staining in most contact lens wearing patients, irrespective of modality, which I consider to be normal. Along these lines, how much physiological corneal staining should we expect to see in a non-problematic contact lens wearer who does not use lens care solutions, such as a continuous lens wearer or daily disposable contact lens wearer?

Dr. Morgan: Most non-contact lens wearers exhibit some level of physiological corneal staining and values up to Grade 1 on the Efron scale (Figure 9) are to be expected.[37]

Dr. Efron: Low grade physiological corneal staining of Grade 1 or less on the Efron scale is a common occurrence among non-problematic contact lens wearers. It is typically transient and will generally resolve within a few hours of ceasing lens wear. In fact, low level staining is intermittently observed in healthy non-lens wearers.

Dr. Karpecki: What level of corneal

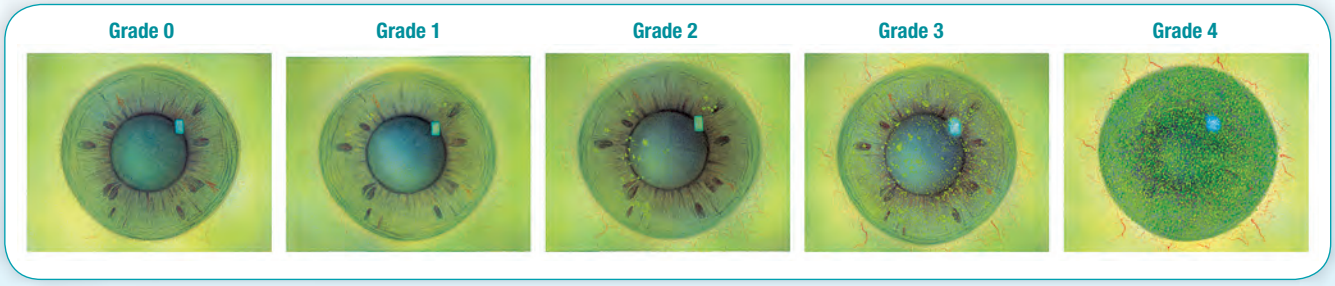

Figure 9. Efron Corneal Staining Grading Scale. A global five step grading system to document the extent and severity of corneal staining (non-PATH). Grade 0 = no staining, not clinically significant, no treatment required; Grade 1 = trace staining, not clinically significant, no treatment required; Grade 2 = mild staining, note in record and watch progression; Grade 3 = moderate staining, therapeutic intervention recommended; Grade 4 = severe staining, treatment required. Reproduced with permission from Nathan Efron and Elsevier. This image was published in "Contact Lens Complications," Second Edition, Nathan Efron, Appendix A, pp. 239-243, copyright Elsevier (2004).

staining differentiates normal physiological staining from pathological staining and requires clinical intervention?

Dr. Morgan: The etiology of the staining is key here, but in general, non-transient Grade 2 or higher might require some form of management or intervention.

Dr. Efron: As a general rule, non-transient corneal staining of Grade 2 or more on the Efron scale is considered as being clinically significant. Practitioners should try and identify the cause of any observed staining, after discounting the possibility of PATH, so that action can be taken to avoid future occurrences. However, the cause of corneal staining is not always obvious. Certainly, if persistent Grade 2 or greater corneal staining is observed, especially if associated with discomfort and/or limbal redness, then clinical intervention is required. In the first instance, a temporary cessation of lens wear is indicated, which in most cases will lead to a resolution of the problem.

Dr. Karpecki: If you were advising one of our colleagues, what would you recommend they do if they see excessive PATH?

Dr. Morgan: Eye care practitioners should expect to see some staining-type response with lens/solution combinations, especially after a short time of lens wear. If staining is present beyond what might be routinely expected, then practitioners should consider causes other than a simple lens/solution one and act accordingly.

Dr. Efron: Just sit back and enjoy the pretty light show! Nothing needs to be done, as PATH staining is benign. However, practitioners should remain alert, as the possibility exists that PATH might be masking corneal staining due to a contact lens complication, and have an ongoing discourse with patients about their satisfaction with their lenses and whether they are experiencing any signs or symptoms that would be indicative of an underlying problem. With our increased understanding of the time-course of these preservative-associated phenomena, practitioners should be able to differentiate PATH from pathological corneal staining with confidence.

Dr. Karpecki: I want to thank my colleagues for a lively and informative discussion. Based on the research presented and our exchange, I feel that these new findings have begun to explain the disconnect in contact lens wearers who use MPS and what we were observing under the slit-lamp and the lack of signs and symptoms that would be indicative of a complication.

While a significant effort has been made to connect PATH with adverse outcomes over the past decade, to date, no negative sequelae have been shown to be associated with PATH. It is essential that we, as clinicians, understand the tools in our armamentarium so with proper use, we can understand what we are observing in our patients and why. It is only then that we can make the best clinical decisions for our patient's health and satisfaction. ✪

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