Human relevant approaches to assess eye irritation: Overview and comparative analysis of test methods

Webinar Series on the

Use of New Approach Methodologies (NAMs) in Risk Assessment

Acceptance and Use of In Vitro and Ex Vivo Eye Irritation Test Methods

3 November 2021

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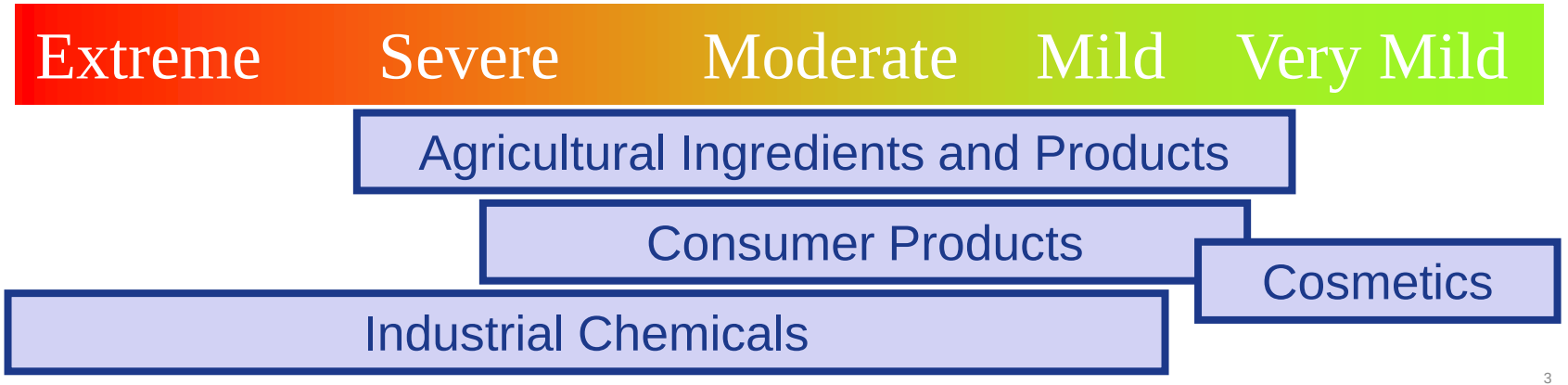
Hans Raabe, VP, COO

Overview

- Overview of the Draize eye irritation test
- Corneal physiology and overview of eye irritation events of concern
- Depth of Injury Concept
- Mechanistic relevance of available non-animal test methods for eye irritation assessment

How have we traditionally conducted testing?

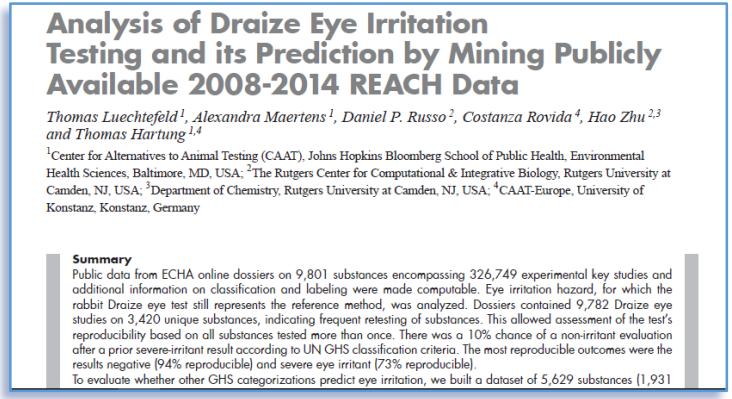
EPA I	EPA II	EPA III	EPA IV
Rabbit Draize Test			
GHS 1	GHS 2	Non-classified	



Draize Rabbit Eye Test Method

- Primary *in vivo* method (developed in 1944)
- Accepted by numerous agencies globally
- Test substance placed in lower conjunctival sac
- Cornea, Iris, Conjunctiva evaluated
- Animal observed over 21 days for apical events
- Conservative/hazard assessment – given differences between human and rabbit eyes
- Subjectivity and Variability

Reproducibility of the Draize Eye Test



Analysis of Draize Eye Irritation Testing and its Prediction by Mining Publicly Available 2008-2014 REACH Data

Thomas Luechtefeld [1], Alexandra Maertens [1], Daniel P. Russo [2], Costanza Rovida [4], Hao Zhu [2,3] and Thomas Hartung [1,4]

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Summary

Public data from ECHA online dossiers on 9,801 substances encompassing 326,749 experimental key studies and additional information on classification and labeling were made computable. Eye irritation hazard, for which the rabbit Draize eye test still represents the reference method, was analyzed. Dossiers contained 9,782 Draize eye studies on 3,420 unique substances, indicating frequent retesting of substances. This allowed assessment of the test's reproducibility based on all substances tested more than once. There was a 10% chance of a non-irritant evaluation after a prior severe-irritant result according to UN GHS classification criteria. The most reproducible outcomes were the results negative (94% reproducible) and severe eye irritant (73% reproducible).

To evaluate whether other GHS categorizations predict eye irritation, we built a dataset of 5,629 substances (1,931

Prior type	1	2A	2B	NC	Total
1	73%	16.1%	0.4%	10.4%	46
2A	4.2%	32.9%	3.5%	59.4%	138
2B	0.2%	4%	15.5%	80.2%	86
NC	1.1%	3.5%	1.5%	93.9%	400

- ECHA database evaluation (UN GHS categories)
- 491 substances with at least 2 Draize eye studies
- Conditional probabilities of Draize evaluations based on a previous test result
- Ex: 46 substances had multiple Draize test results that included at least one Category 1 response

Reproducibility of the Draize Eye Test

Prior type	1	2A	2B	NC	Total
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2B	0.2%	4%	15.5%	80.2%	86
NC	1.1%	3.5%	1.5%	93.9%	400

Most reproducible results were at the extremes

- 94% likelihood to confirm a NC prediction
- 73% likelihood to confirm a severe (GHS 1) prediction
- 10.4% of Category 1 materials predicted as NC in a subsequent test

Luechtefeld et al., ALTEX 33(2), 2016.

Reproducibility of the Draize Eye Test

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- Category 2A and 2B more likely to be NC than Category 2 in a subsequent test
- Minimal discrimination between Category 2B and NC

  (77 of 86 substances with at least one GHS 2B result also have at least one NC prediction)
- NICEATM is now curating available rabbit eye test data to repeat this analysis (for GHS categories) and to also evaluate EPA categories

Luechtefeld et al., ALTEX 33(2), 2016.

Sources of Test Method Variability

Parameters	Draize Eye Test	Non animal methods
Dosing	Dose volume may overfill cul-de-sac Spill-out commonly reported	Precise control of dose applied (±2%) No loss of dose during exposure
Exposure time	Actual exposure times variable due to spill and animal blinking/pawing	Precise control of exposure period, and dose rinse-out timing
Test system	Animal behaviors (pawing, blinking, rubbing) may affect dosing and endpoint expression; Variability among replicates	Test system conditions tightly controlled between replicates Consistency among replicates
Endpoints	Subjective apical observations	Objective machine-read data

Using mechanistic information and human relevance

Consider strengths and limitations of all available methods with respect to:

- their relevance to human ocular anatomy
- the mechanisms of eye irritation/corrosion in humans

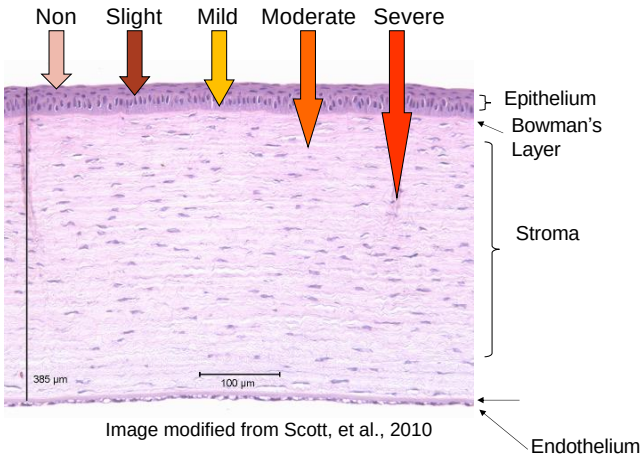

Image modified from Scott, et al., 2010

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REVIEW ARTICLE

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Human-relevant approaches to assess eye corrosion/irritation potential of agrochemical formulations

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ABSTRACT

There are multiple *in vitro* and *ex vivo* eye irritation and corrosion test methods that are available as internationally harmonized test guidelines for regulatory use. Despite their demonstrated usefulness to a broad range of substances through inter-laboratory validation studies, they have not been widely adopted for testing agrochemical formulations due to a lack of concordance with parallel results from the traditional regulatory test method for this endpoint, the rabbit eye test. The inherent variability of the rabbit test, differences in the anatomy of the rabbit and human eyes, and differences in modelling exposures in rabbit eyes relative to human eyes contribute to this lack of concordance. Ultimately, the regulatory purpose for these tests is protection of human health, and, thus, there is a need for a testing approach based on human biology. This paper reviews the available *in vivo*, *in vitro* and *ex vivo* test methods with respect to their relevance to human ocular anatomy, anticipated exposure scenarios, and the mechanisms of eye irritation/corrosion in humans. Each of the *in vitro* and *ex vivo* methods described is generally appropriate for identifying non-irritants. To discriminate among eye irritants, the human three-dimensional epithelial and full thickness corneal models provide the most detailed information about the severity of irritation. Consideration of the mechanisms of eye irritation, and the strengths and limitations of the *in vivo*, *in vitro* and *ex vivo* test methods, show that the *in vitro/ex vivo* methods are as or more reflective of human biology and less variable than the currently used rabbit approach. Suggestions are made for further optimizing the most promising methods to distinguish between severe (corrosive), moderate, mild and non-irritants and provide information about the reversibility of effects. Also considered is the utility of including additional information (e.g. physical chemical properties), consistent with the Organization for Economic Cooperation and Development's guidance document on an integrated approach to testing and assessment of potential eye irritation. Combining structural and functional information about a test substance with test results from human-relevant methods will ensure the best protection of humans following accidental eye exposure to agrochemicals.

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KEYWORDS
Eye irritation; eye corrosion; EPA; agrochemicals; human-relevant; *in vitro*; BCOP; EpiOcular; ICE; neutral red release

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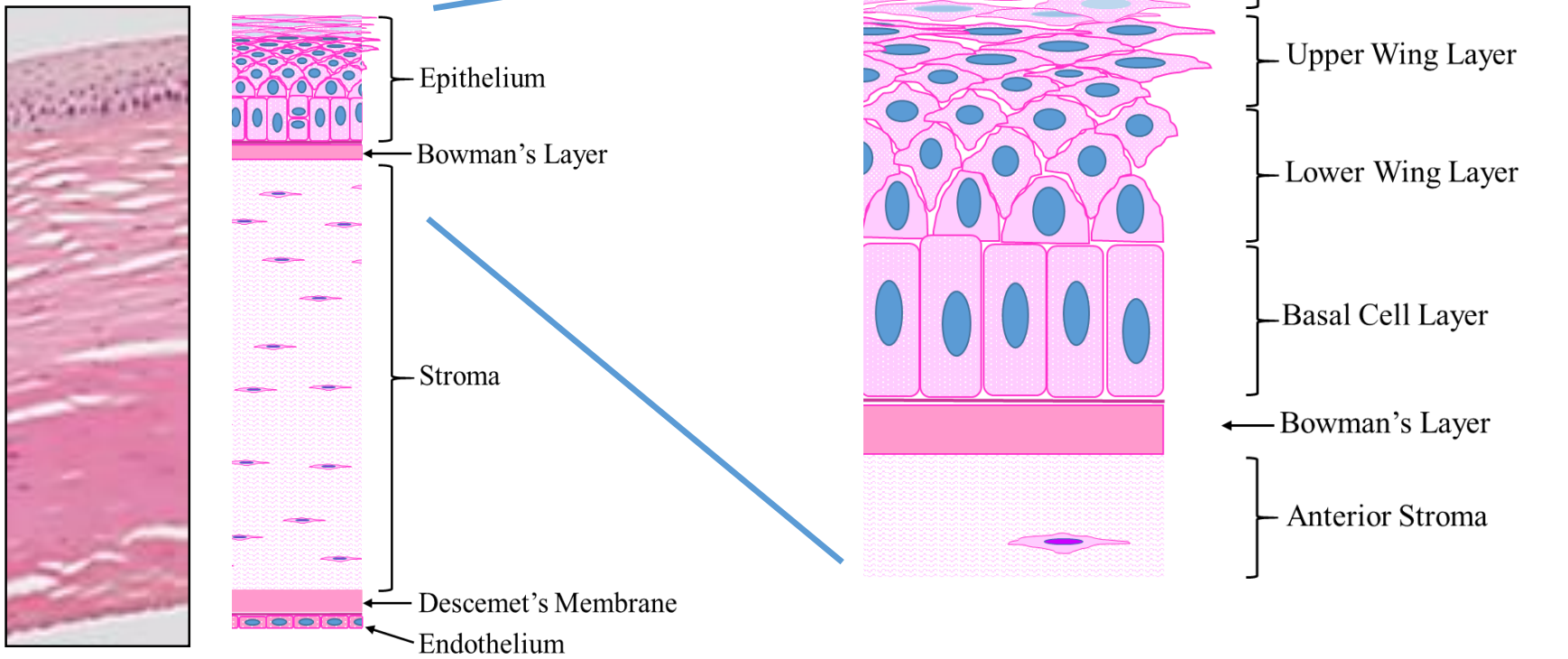
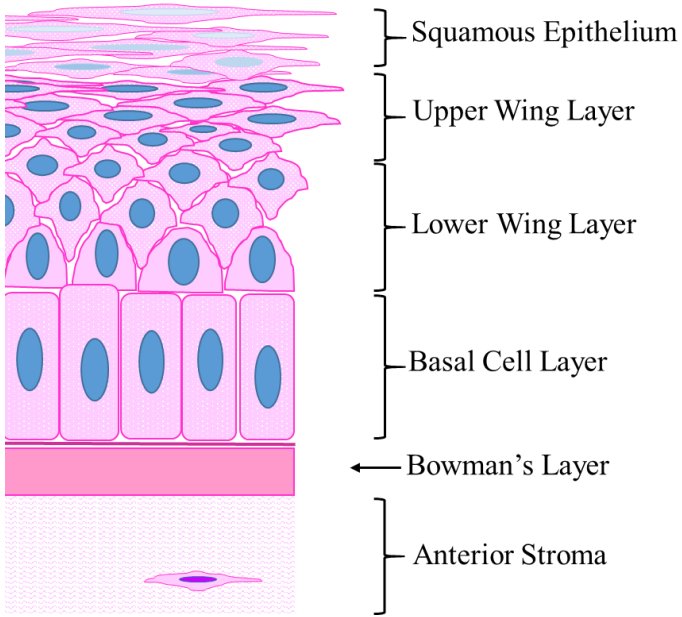
Corneal Physiology and Tissue Functions



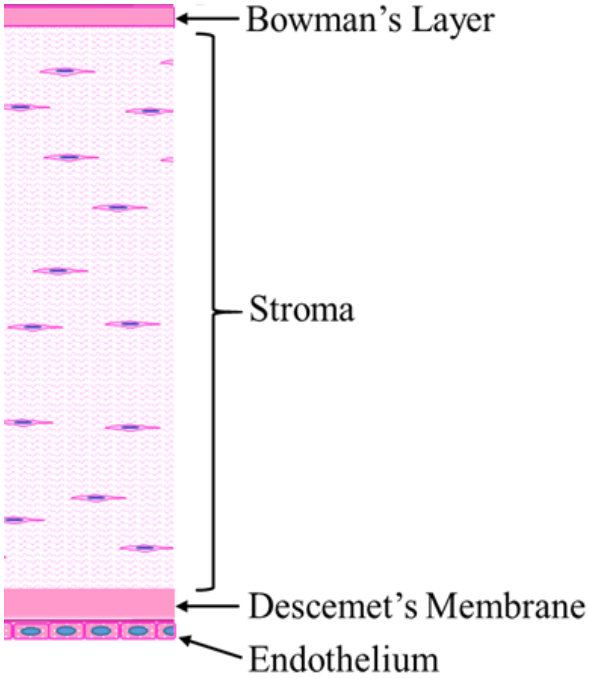
Corneal Physiology and Tissue Functions



Epithelium

- Protection from xenobiotic and foreign material insults
- Provides an optical interface
- Maintains ideal stromal hydration state
- Bowman's Layer and basal membrane provide structure and matrix for basal cell layer
- Basal cells – proliferative cells maintain basal layer matrix; are source for upward epithelial development and stratification; corneal wound healing through sheet migration and rapid proliferation
- Wing cells – intermediate cells expressing precursors of tight junctions; provide significant structural support
- Squamous cells – protective barrier / zona occludens

Corneal Physiology and Tissue Functions



Stroma and Endothelium

- Stroma: makes up 80% of the corneal cross-section
- Optical clarity and light transmission functions
- Keratocytes – sparse but networked cells involved in maintenance of organized collagen fiber bundles
- Disorganized collagen fibers result in opacities
- Disruption of keratocytes induces inflammatory response to stimulate keratocyte proliferation, migration and reestablishment of collagen fibers
- Descemet's Membrane provides structure and anchoring matrix for endothelial cell layer
- Endothelium –non-proliferative single cell layer maintains ideal stromal hydration

Depth of Corneal Injury Concept

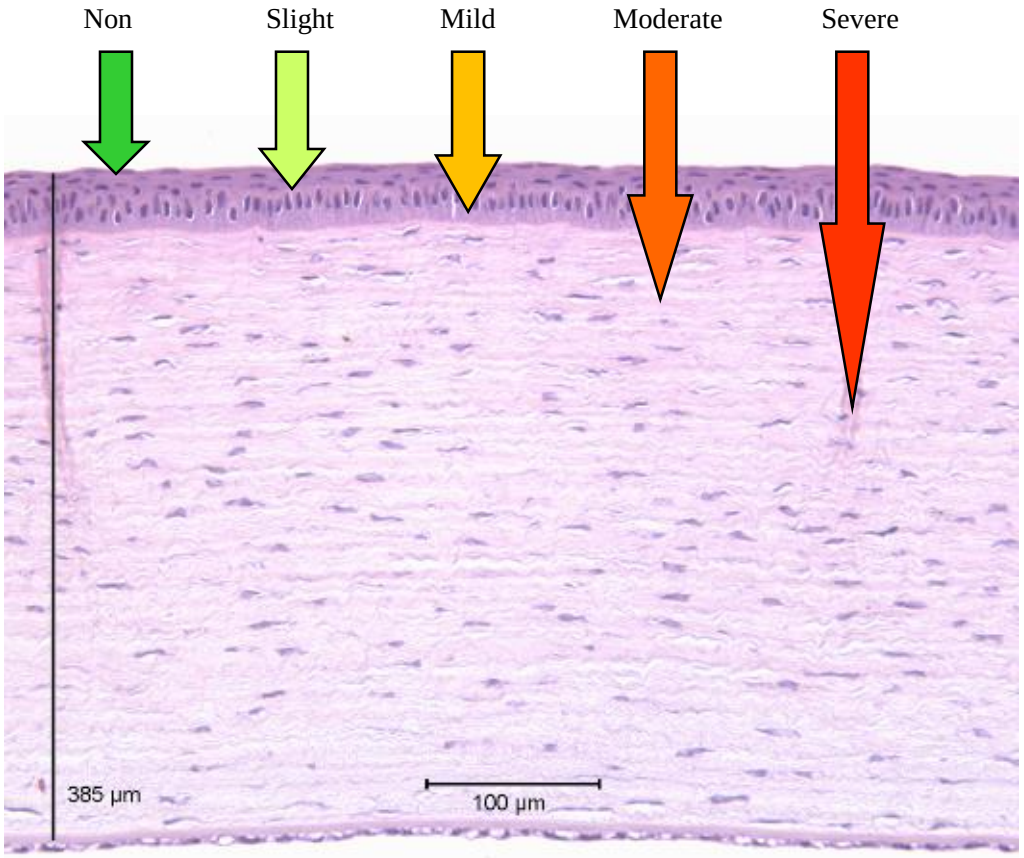
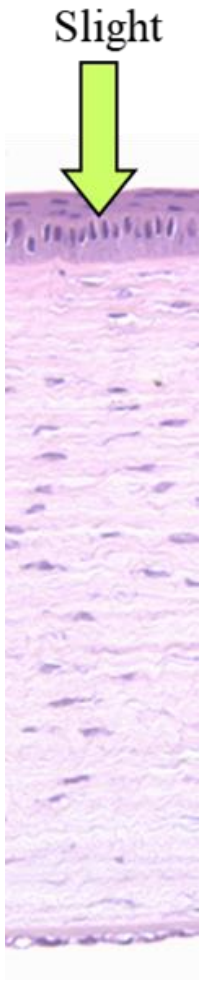


Image modified from Scott, et al., 2010

Depth of injury is predictive of the degree and duration of injury

"Regardless of the process leading to tissue damage, extent of initial injury is the principal, mechanistic factor determining the outcome of the ocular irritation"

Maurer et al, 2002



Damage Limited to the Superficial Conjunctival or Corneal Epithelium

CELLULAR RESPONSE

Upon exposure to the squamous epithelium, chemicals may induce

- cell stress responses
- release of chemokines and cytokines
- changes in relevant biomarkers
- breakdown of the tight junctions
- loss of cell to cell adhesion molecules
- changes in cell metabolism/respiration
- necrotic or apoptotic damage

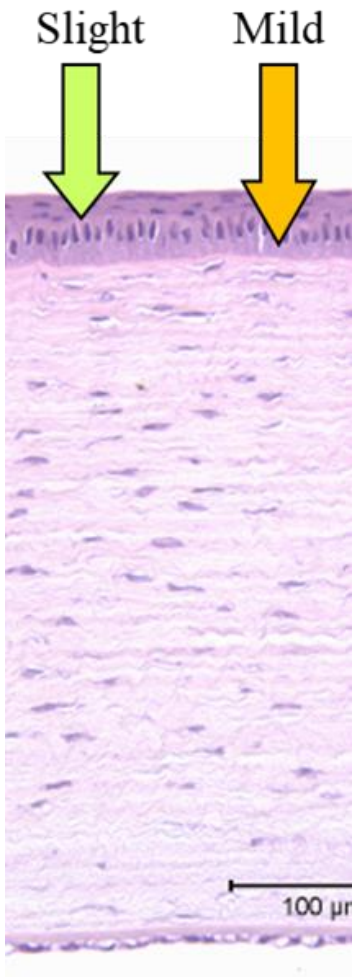
- **epithelial cell death**

ORGAN RESPONSE

- increased corneal or conjunctival permeability/loss of barrier function
- susceptibility to xenobiotics
- conjunctival hyperemia and discharge
- swelling of the conjunctival tissues
- transient and mild corneal swelling

- sloughing of superficial epithelial cells
- induction of wound healing response and basal cell regeneration/turnover
- limited inflammatory response and neutrophil migration

Rapid recovery of the corneal and conjunctival tissues typical



Damage Limited to the Wing Cell Layer of the Epithelium

CELLULAR RESPONSE

Upon penetration into the squamous epithelium and upper wing cells, or the conjunctival layers, chemicals may induce

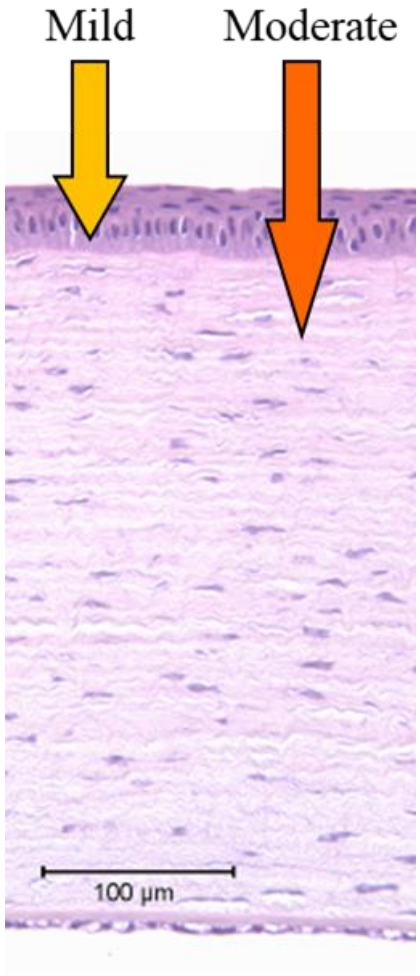
- cell stress responses
- release of chemokines and cytokines
- changes in relevant biomarkers
- breakdown of the tight junctions
- damage to the desmosomes
- loss of cell to cell adhesion molecules
- changes in cell metabolism/respiration
- necrotic or apoptotic damage

- **cell death**

ORGAN RESPONSE

- increased corneal permeability/loss of barrier function
- Increased susceptibility to xenobiotics
- corneal swelling and related opacity
- corneal opacity due to cellular/molecular denaturation/coagulation
- sloughing of mid to lower epithelial tissues
- increased induction of wound healing response and basal cell regeneration/turnover
- increased potential for inflammatory response and neutrophil migration

Recovery of the corneal and conjunctival tissues likely



Damage Into The Lower Wing Cell and Basal Cell Layers

CELLULAR RESPONSE

Upon penetration into the lower wing cells, and/or into the basal cell layers, chemicals may induce

- cell stress responses
- release of chemokines and cytokines
- loss of cell to cell adhesion and cell to basement membrane adhesion
- changes in cell metabolism/respiration
- necrotic or apoptotic damage
- **cell death**
- changes in basement membrane? *

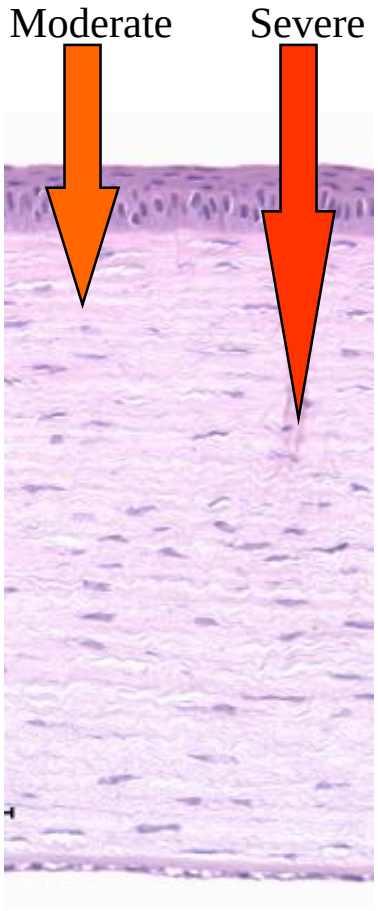
ORGAN RESPONSE

- increased corneal permeability/loss of barrier function
- susceptibility to xenobiotics
- corneal swelling and related opacity
- corneal opacity due to cellular/molecular denaturation/coagulation
- sloughing of lower epithelial tissues
- increased induction of wound healing response and basal cell regeneration/turnover increased
- inflammatory response and neutrophil migration

Recovery of the corneal tissues expected but prolonged.

* Basement membrane integrity is essential

Damage Into the Corneal Stroma



CELLULAR RESPONSE

Upon penetration through the epithelium into the corneal stroma, chemicals may induce

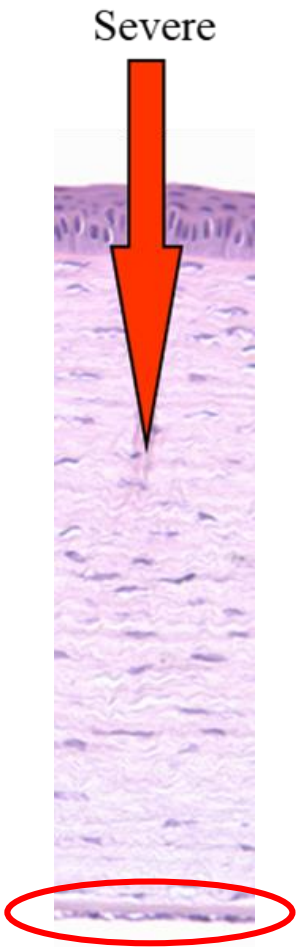
- cell stress responses
- retraction of keratocyte cell to cell network
- release of chemokines and cytokines, primarily IL-1α and TNFα
- induction of extracellular matrix / collagen synthesis
- activation of matrix metalloproteases result in loss of cell to cell adhesion and local tissue restructuring
- changes in cell metabolism/respiration
- necrotic or apoptotic damage

- **Keratocyte cell death**

ORGAN RESPONSE

- susceptibility to xenobiotics
- progressive ulceration and tissue necrosis
- notable stromal swelling and related opacity
- corneal opacity due to cellular/molecular denaturation/coagulation
- induction of wound healing response and basal cell regeneration/turnover
- recruitment of neutrophils / inflammatory response in stroma
- fibrosis resulting in disorganized collagens
- pannus and neovascularization
- loss of endothelium

Recovery becomes less likely with progression of the depth and degree of injuries



Damage involving the Corneal Endothelium

CELLULAR RESPONSE

Upon penetration through the corneal epithelium and stroma, chemicals may induce

- cell stress responses, leading to changes in cell adhesion
- release of chemokines and cytokines
- changes in relevant biomarkers
- activation of matrix metalloproteases result in loss of cell to cell adhesion and cell to Descemet's membrane adhesion
- changes in cell metabolism/respiration
- necrotic or apoptotic damage

- **Endothelial cell death**

ORGAN RESPONSE

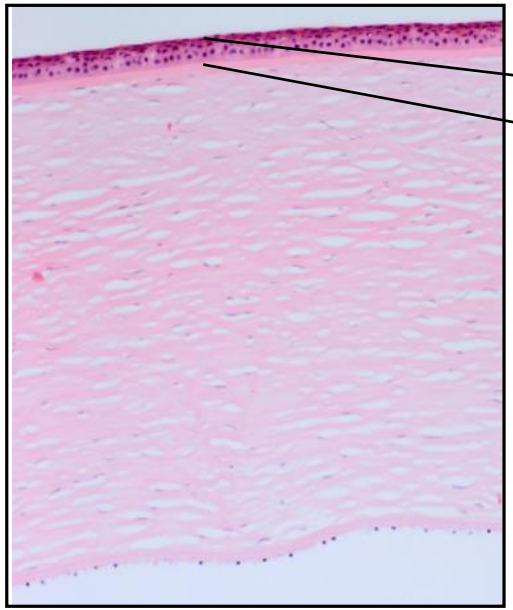
- notable lower corneal swelling and swelling-related corneal opacity
- loss of endothelium
- loss of keratocytes in lower stroma

No meaningful recovery of cornea

Test Method Relevance to Corneal Cross-sections

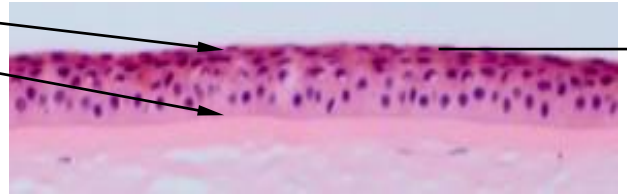
Full thickness Cornea

epithelium, stroma and endothelium



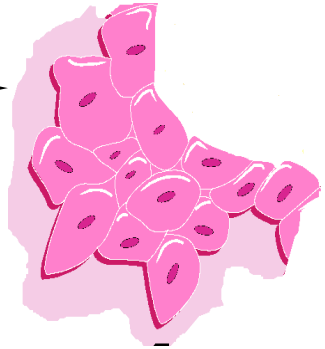
Epithelium

Squamous, wing, and basal cells



Squamous Epithelium

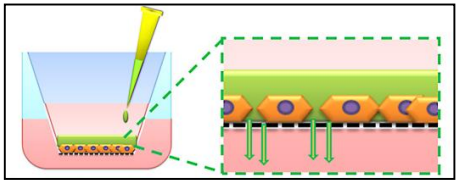
Outermost cells covering epithelium



Available non-animal test methods model different portions of the cornea.

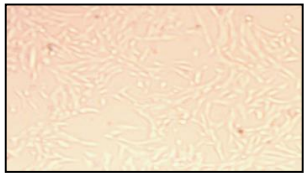
Its important to understand the relationship of those test methods to the various corneal layers to appreciate the mechanistic relevance in eye irritation assessments.

Fluorescein Leakage Assay

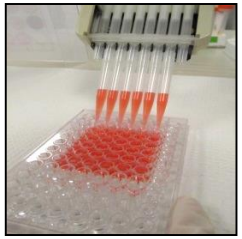


https://www.researchgate.net/figure/Schematic-diagram-of-the-Fluorescein-Leakage-FL-assay_fig5_268446464

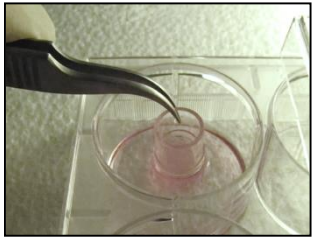
Squamous epithelium



Short Time Exposure Assay

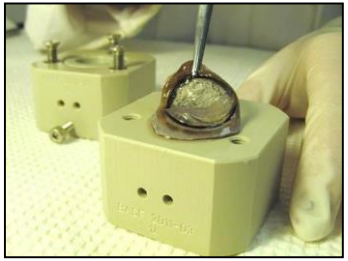


Reconstructed Human Cornea-like Epithelium Test

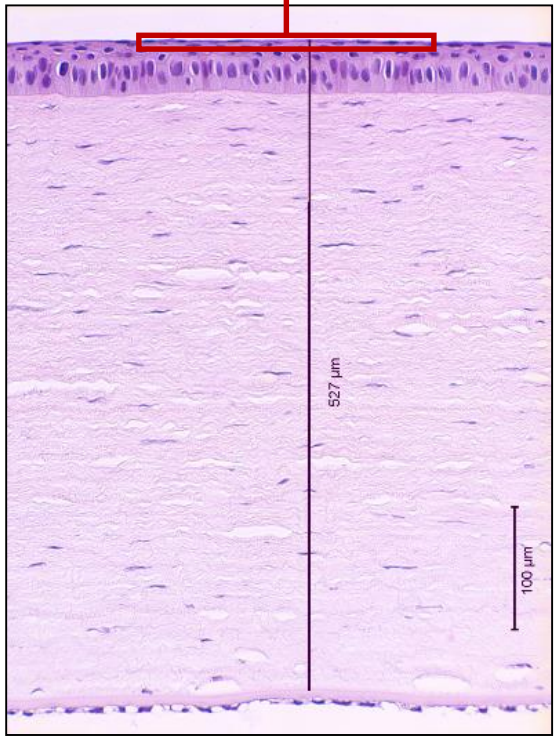


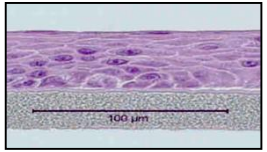
Full thickness corneal models

Bovine Corneal Opacity and Permeability Assay



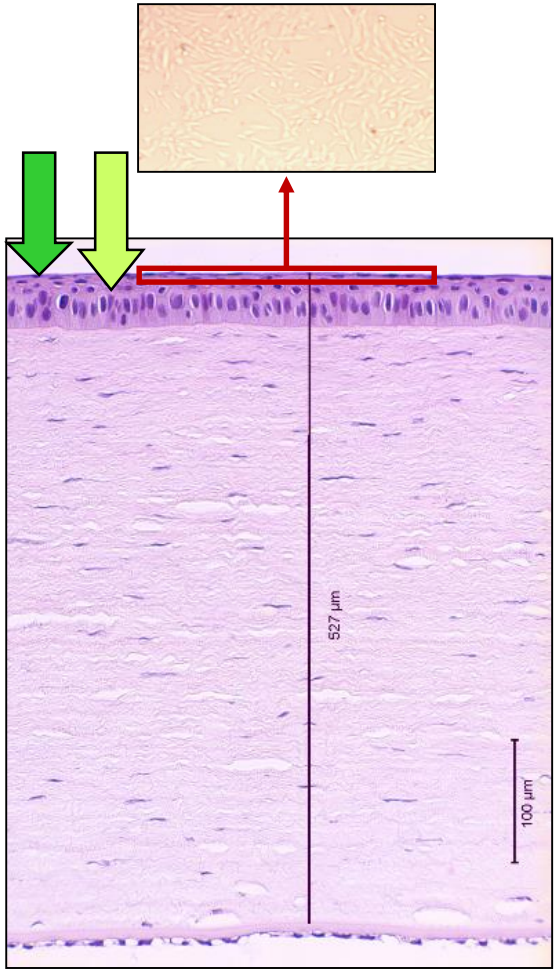
Isolated Chicken Eye Test





Epithelium models

Squamous epithelium models



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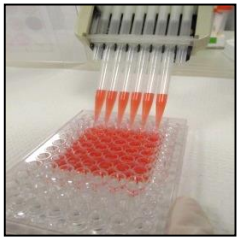
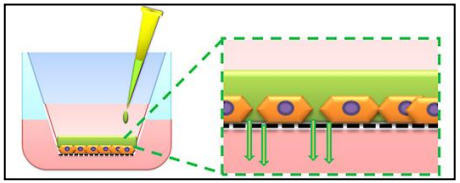
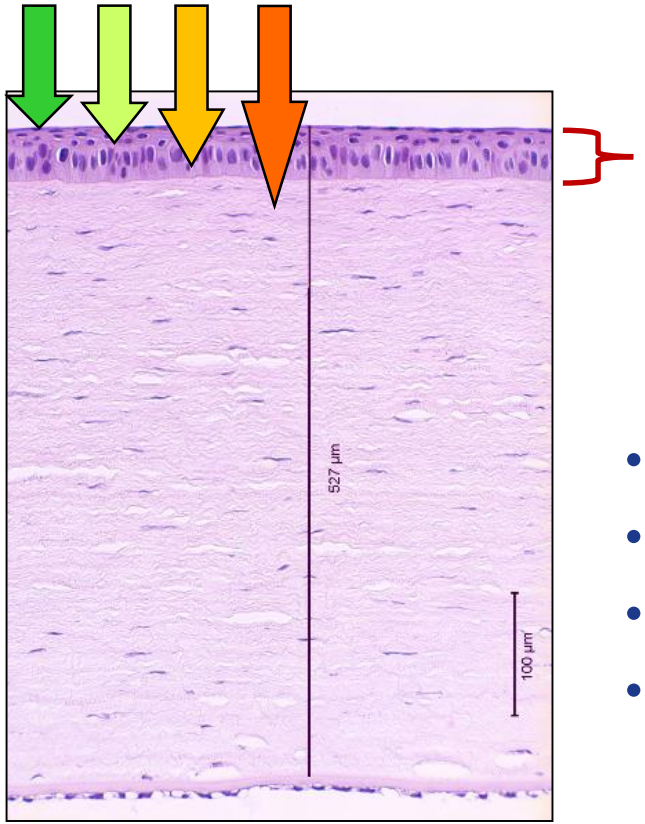


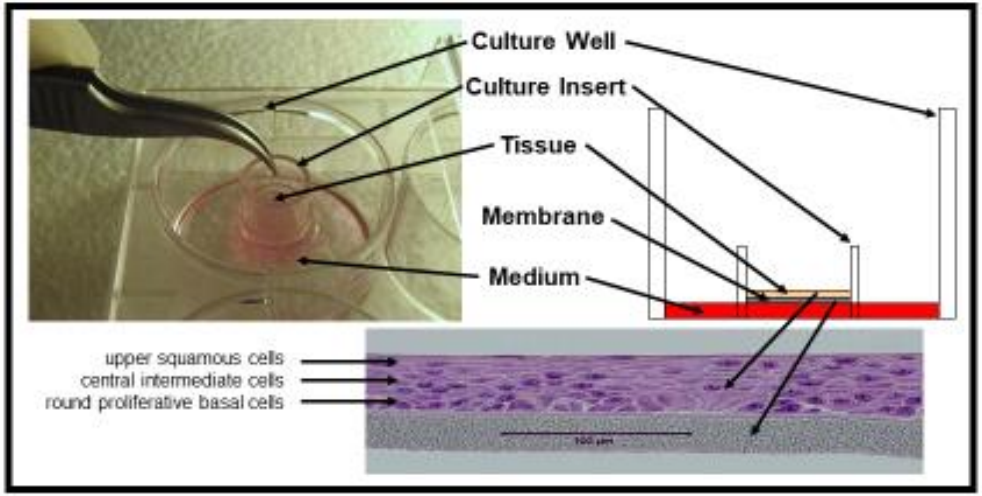
Fluorescein Leakage Assay



- Model the upper-most squamous layer
  - Relevant to tight junction and barrier disruption
  - Validated methods do not use human cells
- Cell viability / cell death can be determined
- Concentration-based prediction models correlate to severe and/or non-irritants
- Depth of injury not modeled
  - Mechanistically limited to discriminating non-irritants from irritants

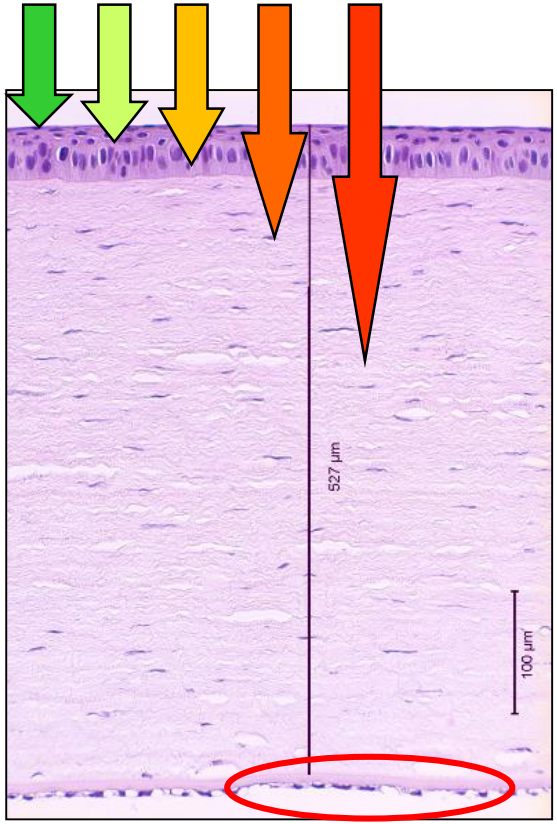
Reconstructed human corneal epithelium models





- Model the stratified human corneal epithelium
- Cell viability / cell death are determined
- Cytokine release / expression can be measured
- Depth of injury into epithelium modeled
  - Discriminate among non, mild and moderate irritants

Full corneal thickness models



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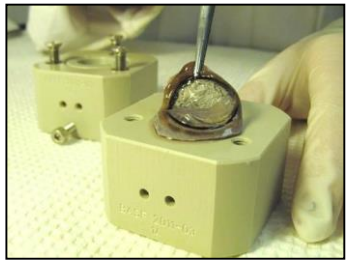
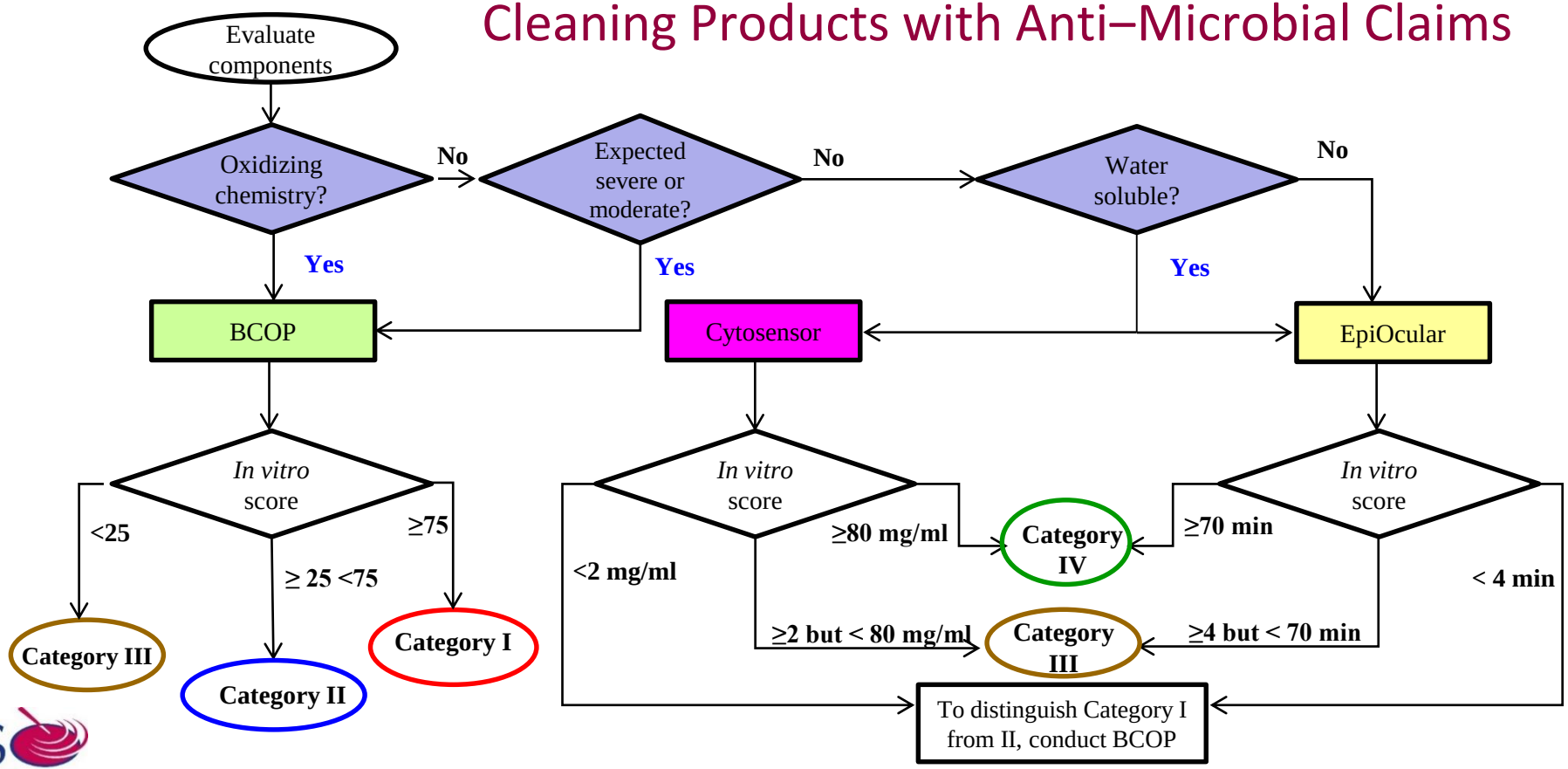


Isolated Chicken Eye Test

- Model all layers of the cornea
  - non-human species used; human eyes are rare
- Opacity, swelling, loss of barrier measured
- Histopathology can be very helpful for DOI
- Other endpoints possible (viability, cytokine)
- Model penetration and injury in all corneal layers
  - Discriminate among all categories

EPA OPP Non-animal Testing Strategy for Cleaning Products with Anti–Microbial Claims



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RhCE Test Method Overview

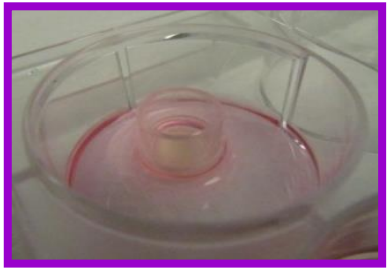
Measuring chemical-induced cell death

Tissue Treatment

Chemicals or formulations are applied without dilution to model real life exposures

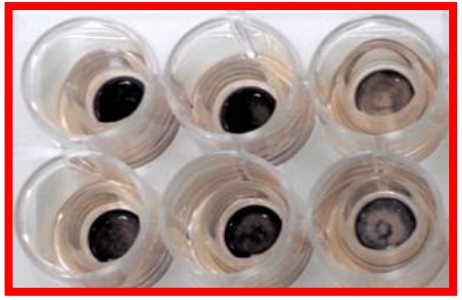
Tissue Rinsing

Post-treatment Expression Incubation

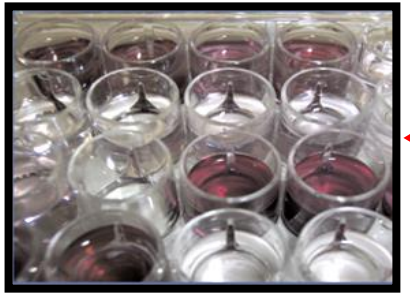


After exposure, tissues are rinsed, immersed in medium for 12 minutes, and then incubated for a post-treatment incubation

MTT Reduction

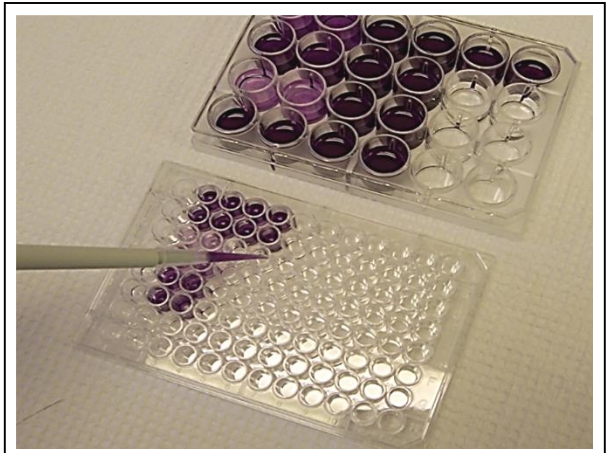


Isopropanol Extraction



Prepare aliquots for spectrophotometry

MTT endpoint for cell cytotoxicity assessment



Extracted MTT is thoroughly mixed and transferred to a 96-well plate.

The 96-well plate/MTT-isopropanol samples are quantified using a microplate reader. Optical Density (OD) at 550 to 570 nm is measured.

OD_{550} values are used to calculate relative viability values.

Viability is presented relative to negative control tissue values

$$\% \text{ of Control} = \frac{\text{Test Material } OD_{550}}{\text{Negative Control } OD_{550}}$$

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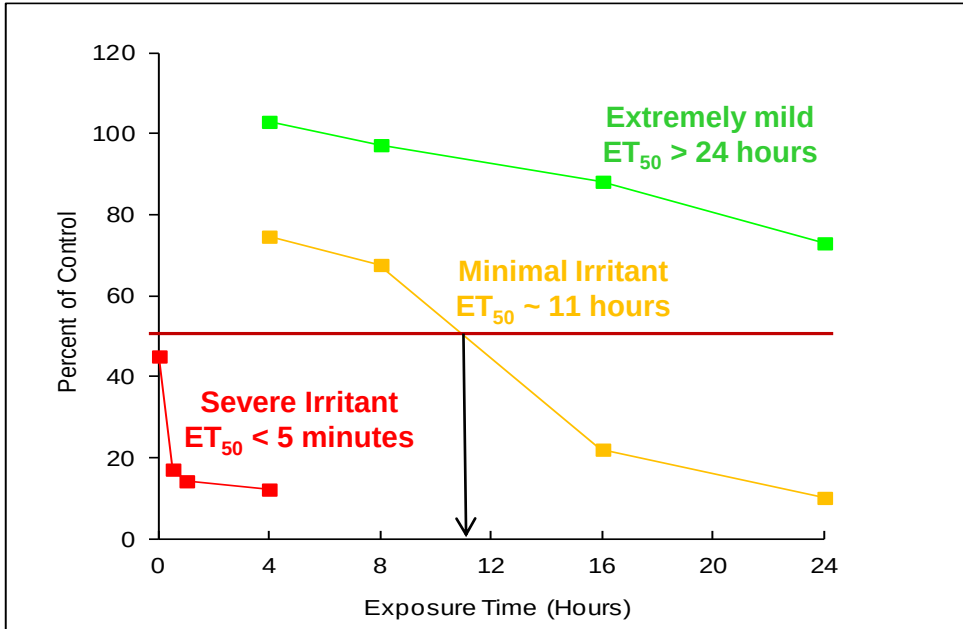
Time-to-toxicity Concept in RhCE Models



ET_{50} (estimated time to reduce viability to 50% of control), plot relative viability over exposure time

US EPA Antimicrobial Cleaning Products (AMCP)

- To discriminate between EPA III and IV or identify EPA Cat I (without further testing)
- Multiple exposure time protocol
- Continuum of responses across eye irritation spectrum
- Also used in product development to create progressively milder/safer formulations
- Rank-order candidate formulations
  - Can include benchmarks for data interpretation

Eye Irritation Test (EIT) Data Evaluation

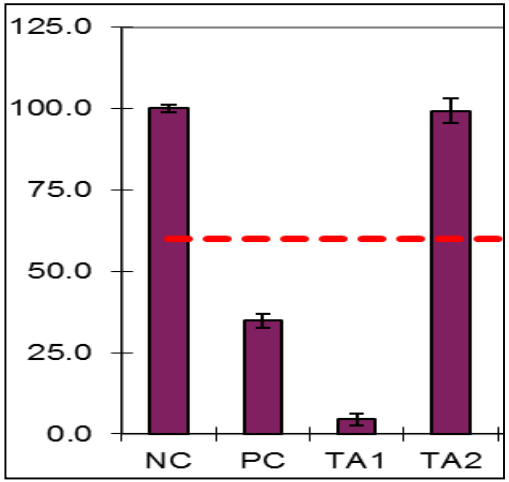
OECD TG 492 for Eye Irritation

Uses a single fixed exposure time (liquids are treated for 30 minutes; solids for 6 hours)

- Viability is assessed by MTT reduction, and the following prediction model applied

For Bottom-up strategy to identify GHS "No Category"

- Viability **> 60%** - test chemical does not require labeling for eye irritation/ serious eye damage (**GHS No Cat**)
- Viability **≤ 60%** - test chemical classified as requiring classification and labelling as an irritant
- **does not distinguish between GHS category 1 or 2 – further testing indicated**



Overall Accuracy	80%
Sensitivity	96%
False Negative Rate	4% !
Specificity	63%
False Positive Rate	37%

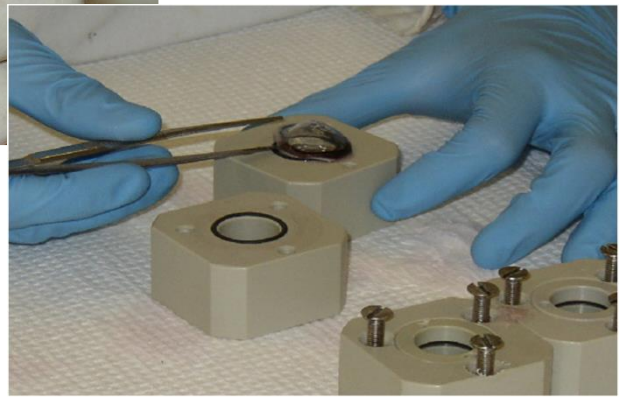
Assay performance when used to identify chemicals that do not induce either moderate or severe eye irritation or damage (GHS No Category)

Bovine Corneal Opacity and Permeability (BCOP) - Overview

Measuring changes in corneal opacity and loss of barrier function

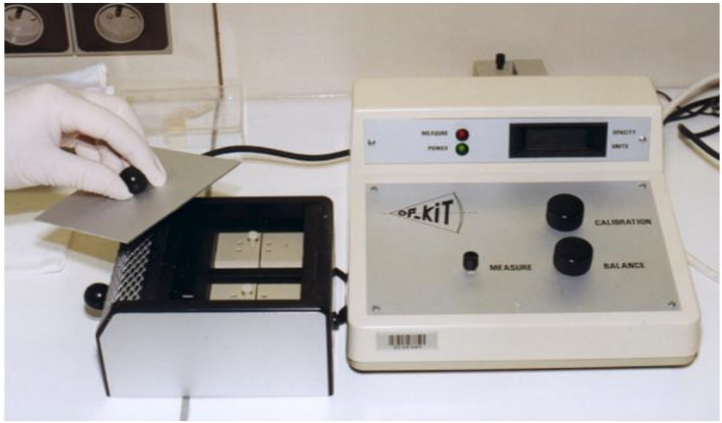
Bovine eyes are obtained as a byproduct of meat production

No live animals used

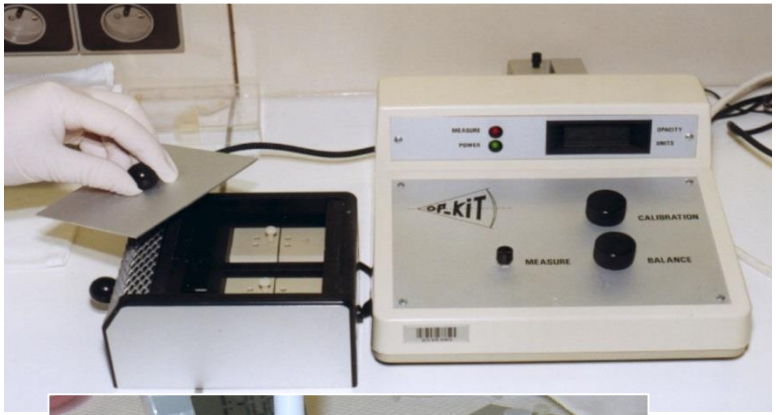


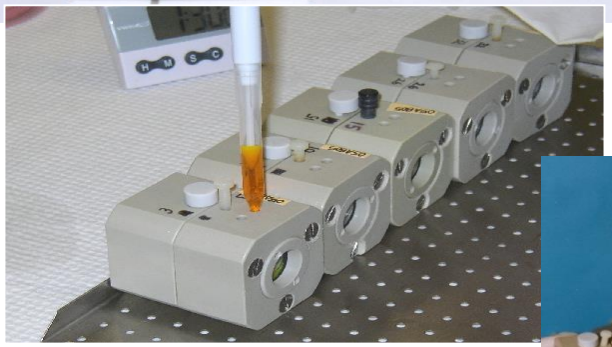
Bovine corneas are mounted in corneal chambers with glass windows. Cultured in EMEM at 32°C

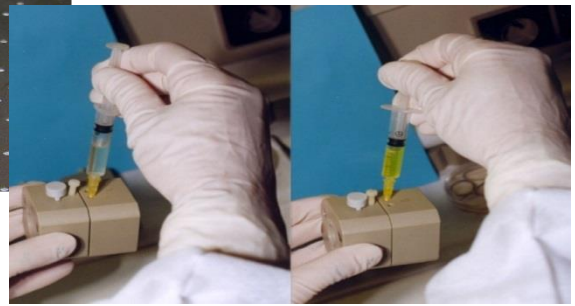
Initial opacity values determined using an opacitometer



Bovine Corneal Opacity and Permeability (BCOP) - Overview







- Treat test chemical
  - 10 minutes (liquids)
  - 4 hours (solids) 20% aqueous preparation
- Rinse / incubate (2 hours for liquids)
  (expression of toxic effects)
- Read post-treatment opacity
- Induction of opacity (up to 150+ units)
- Loss of corneal barrier function

measured by determining fluorescein permeation after 90 minutes (OD_{490})

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BCOP Prediction Models

$$\textit{In Vitro}\ \text{Score} = \text{Opacity} + (15 \times OD_{490})$$

Prediction Model Developed by Merck*
(non regulatory use)

In Vitro Score	Predicted Irritation Potential
≤ 25	Mild
25.1 – 55	Moderate
> 55.1	Severe

Prediction Model per OECD TG 437
(for UN GHS classification and labeling)

In Vitro Score	UN GHS
≤ 3	No Category
>3 ≤ 55	No standalone prediction can be made
> 55	Category 1

The assay provides a continuum of responses across the eye irritation spectrum from mild to severe

*Sina, *et al*. (1995) **Fund. and Applied Tox.** 26:20-31.

Histological Evaluation

Histopathology of progressive surfactant-induced corneal epithelial erosion and stromal swelling.

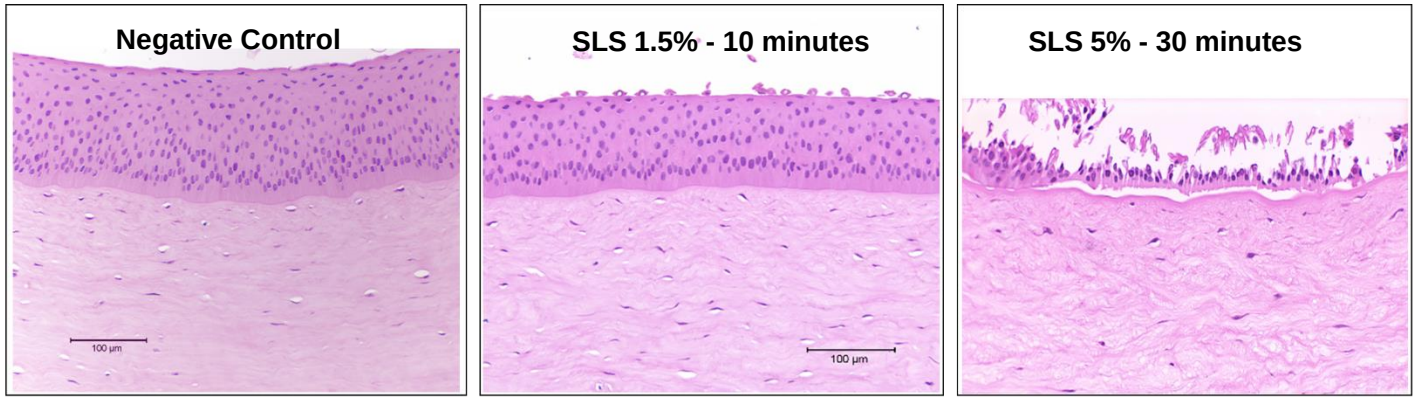


Fig a. Negative Control cornea showing intact epithelium and organized upper stroma.

Fig b. Loss of squamous and upper wing layers, results in increases in FL_{490}.

Opacity = 1.7

FL OD_{490} = 0.302

IVIS = 6.2

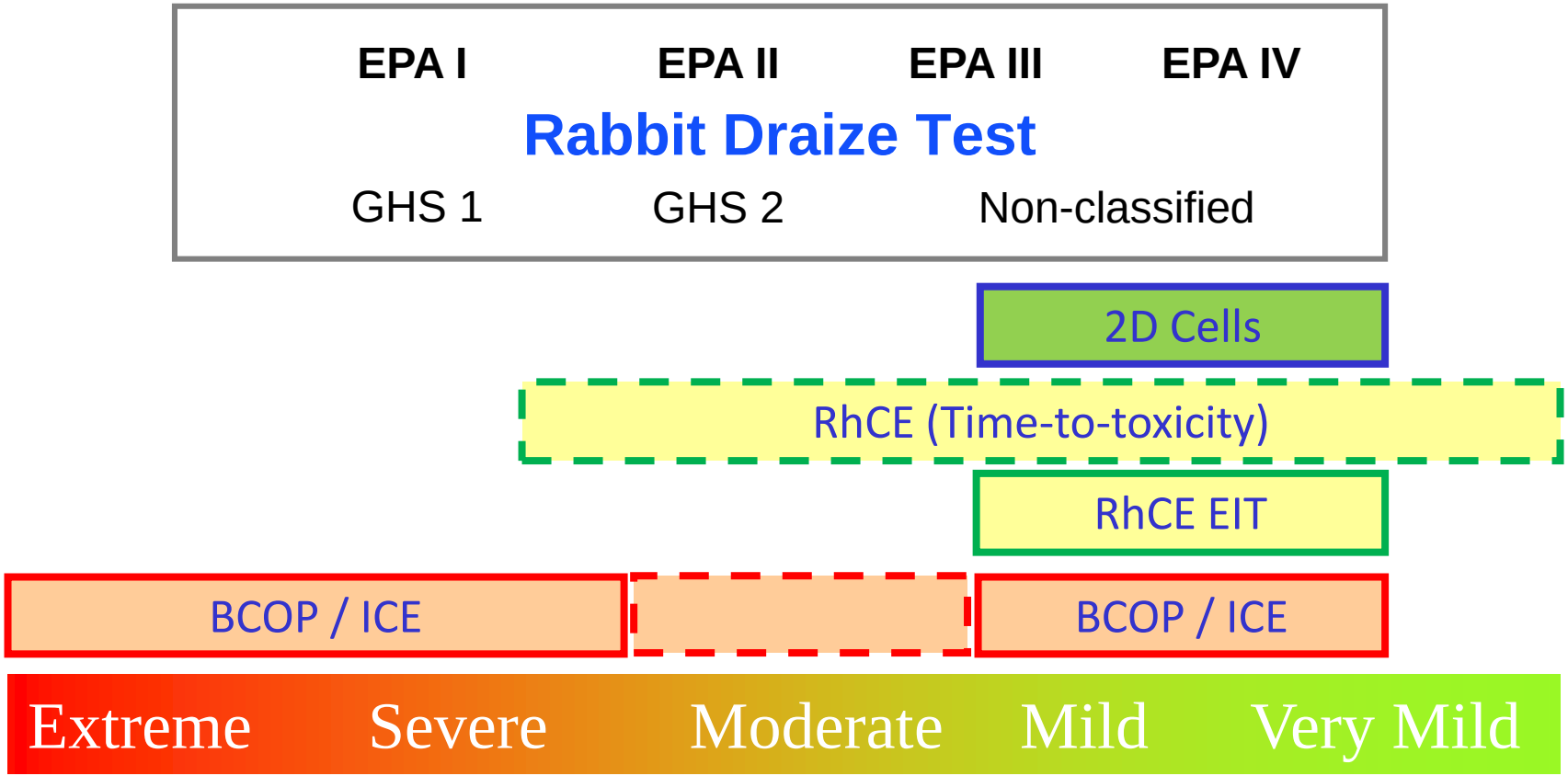
Fig c. Complete loss of epithelium results in high FL_{490}. Marked stromal edema and disorganization results in modest opacity.

Opacity = 7.7

FL OD_{490} = 2.540

IVIS = 45.8

Assays should complement each other
(*integrate mechanisms and evidence*)



Thank You for Your Participation!

For more information on additional assays to address ocular irritation, please visit:

www.iivs.org

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