

# Human 3D corneal models for a detailed quantification of the initial depth of injury as an indicator for cellular damage in the human eye

M. Bartok<sup>1</sup>, D. Gabel<sup>1</sup>, M. Engelke<sup>1</sup>, M. Zorn-Kruppa<sup>2</sup>, J. Brandner<sup>2</sup>, K. Reisinger<sup>3</sup>, K. Daton<sup>3</sup>, K.R. Mewes<sup>3</sup>

<sup>1</sup>Jacobs University Bremen gGmbH, 28759 Bremen, Germany

<sup>2</sup>University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany

<sup>3</sup>Henkel AG & Co. KGaA, 40589 Düsseldorf, Germany

## Abstract

Our study aims at the complete replacement of the Draize Eye Irritation Test by a new test system which is based on biotechnologically produced hemi-cornea equivalents (1). The sophisticated structure of the hemi-cornea model comprises both an epithelium and stroma compartment. Hence, this two-compartment model offers the potential to analyze the initial depth of injury (DOI) after substance application and to discriminate between damages induced in the epithelium and/or the stroma. We present three different approaches for the analysis of the corneal DOI in the hemi-cornea model.

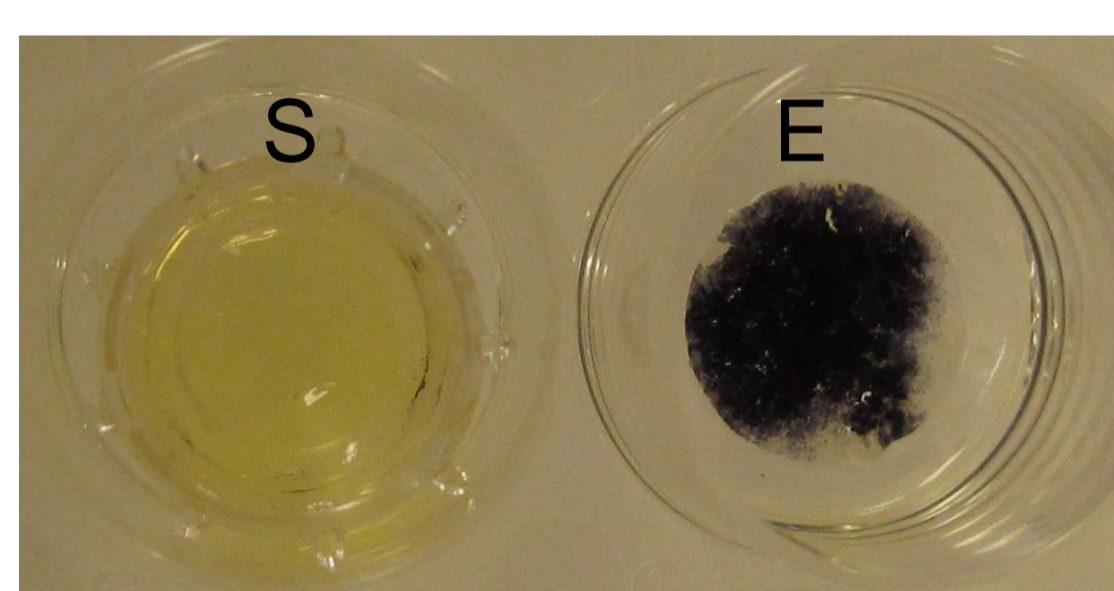
## 1. Insertion of a Bowman's membrane for separation of epithelial layer from the stroma:

### Method

The hemi-cornea models were produced according to the protocol of Engelke et al, 2013, with inserting an artificial Bowman's layer between epithelium and stroma. After topical substance application, the models were incubated with MTT solution and the epithelial layer was separated from the stroma. The two tissues were analyzed separately for cell viability.

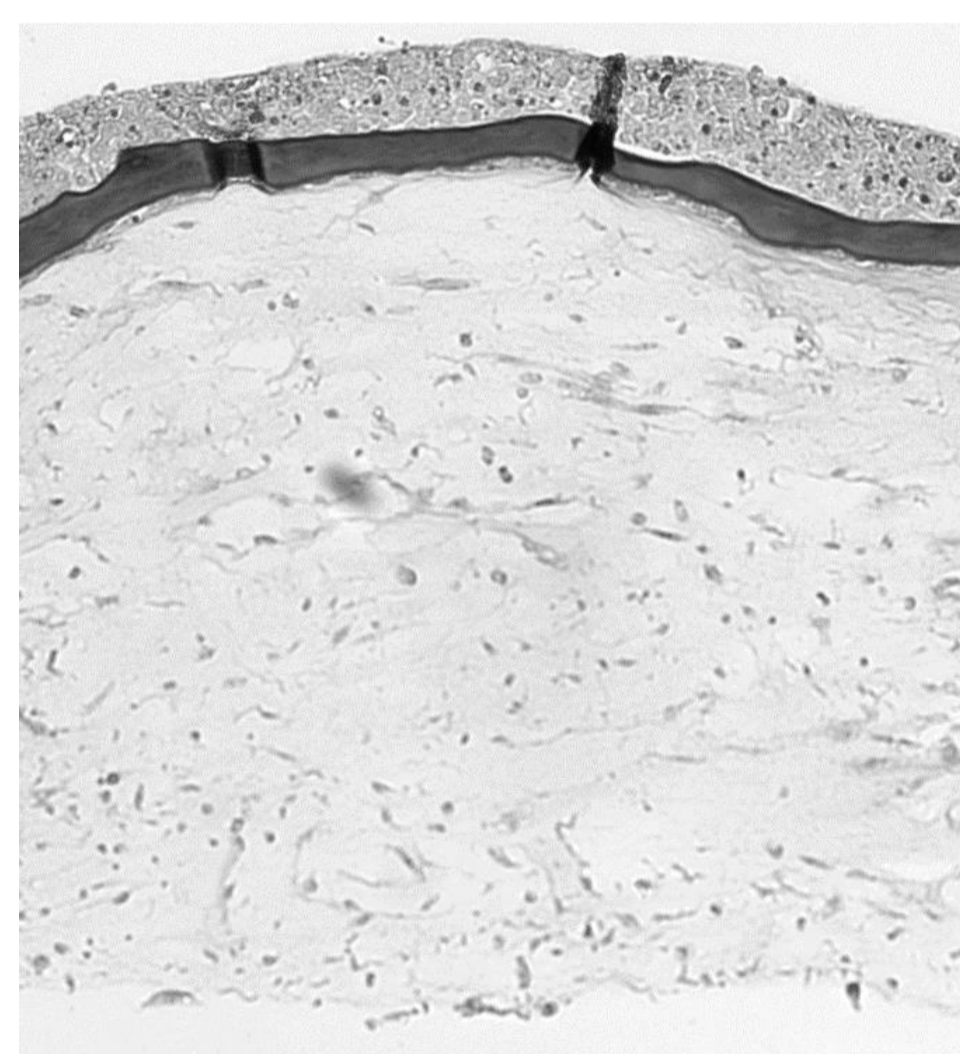
### Results

At first the stroma was prepared without Keratinocytes to demonstrate the complete detachment of the epithelium from the stroma.



**Figure 1:** Hemi-cornea models with separated stroma (S) and epithelium (E) after MTT staining

After the method was proved to be successful, the models were prepared with cells in both compartments for a detailed quantification of the cell viability in both layers separately.



**Figure 2:** Histological section of the hemi-cornea model containing the Bowman's membrane

### Outlook

The new complex 3D hemi-cornea models with inserted Bowman's membrane will be tested with selected reference chemicals for the development of a prediction model. Afterwards, this test system will be prevalidated for reproducibility and predictive capacity by using several reference chemicals from all GHS categories.

## Conclusions

- The DOI analysis using cryo-sections of MTT-stained hemi-cornea models represents a promising tool for the discrimination of all GHS categories and hence, the complete replacement of the Draize Eye Irritation Test in one single test system.
- The insertion of an artificial Bowman's layer allows the separate analysis of the cell viability in stroma and epithelium, which will also contribute to a better discrimination between the 3 GHS categories. This system has to be refined using selected reference chemicals.
- The method of apoptosis detection in the tissue still needs refinement in order to complement the other 2 methods presented here and eventually to increase the overall predictivity of the test strategy.

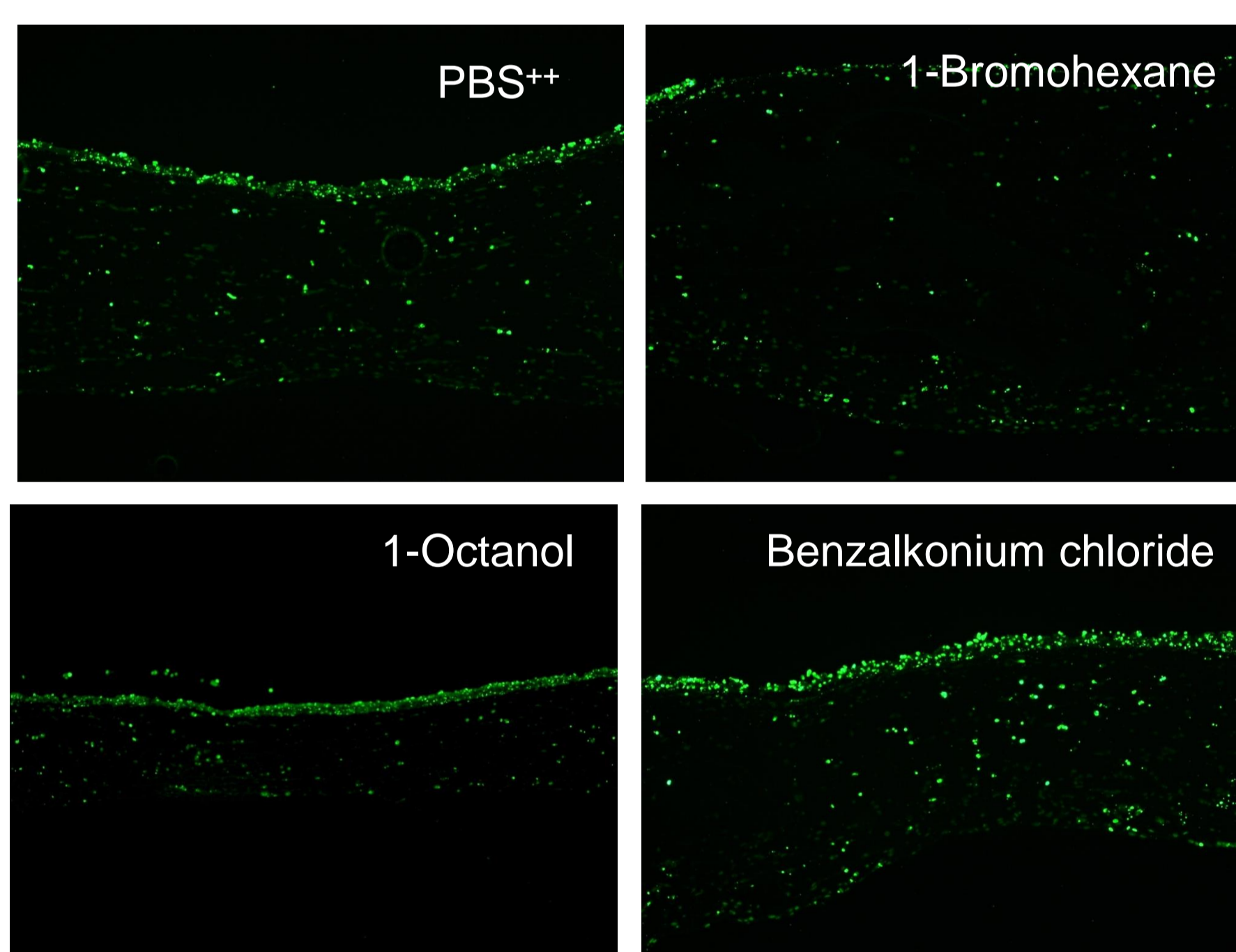
## 2. Detection of apoptotic cells within the hemi-cornea after topical substance application:

### Method

After substance application the hemi-cornea models were fixed, embedded in paraffin and cut into sections of 5 µm thickness. Apoptotic cells were detected by using the TUNEL assay (Roche Diagnostics, Germany). TUNEL-positive cells were identified by their green fluorescence. Chemicals from all GHS categories were compared to PBS treatment as Negative control (NC).

### Results

After substance application TUNEL-positive cells were observed in both epithelium and stroma. The number of TUNEL-positive cells was higher in corneal models treated with GHS category 1 substances compared to tissues treated with non-classified or GHS cat. 2 chemicals. However, even in PBS-treated tissue apoptotic cells were present.



**Figure 3:** PBS: NC; 1-Bromo-hexane: GHS no cat.; 1-Octanol: GHS 2; Benzalkonium chloride 1%: GHS 1

### Outlook

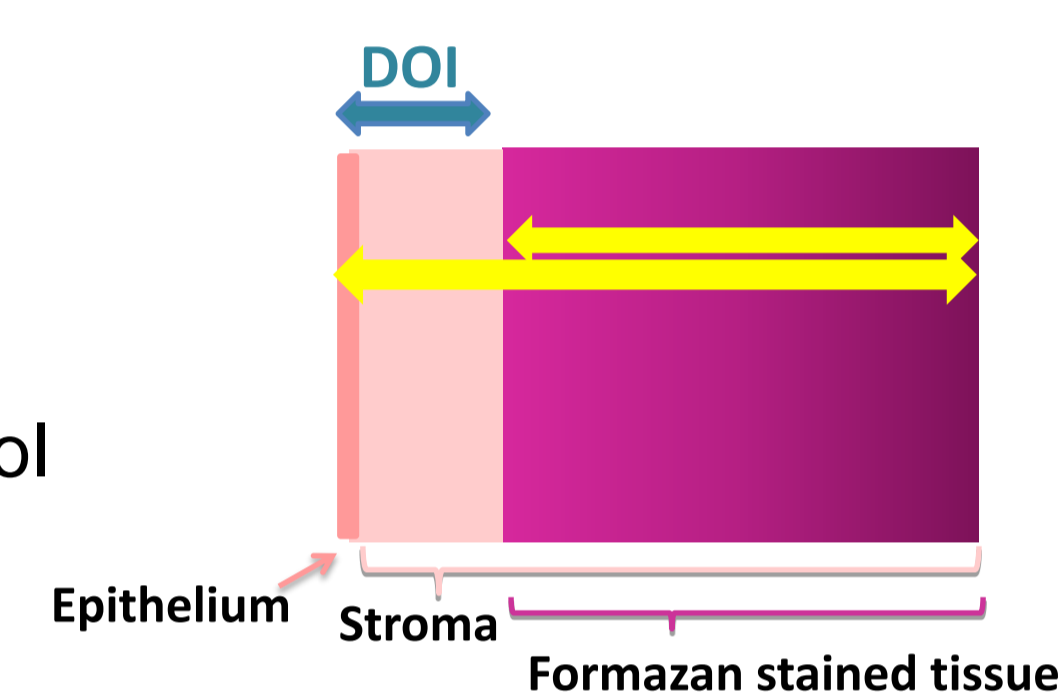
The parameters for substance application and apoptosis detection must be further optimized in order to measure DOI as a reliable endpoint for the prediction of the eye-irritating potential of chemicals. The number of TUNEL-positive cells in the controls must be reduced to draw a clearer distinction between the GHS categories in respect of the DOI.

## 3. Analysis of tissue damage in MTT-stained hemi-cornea models:

### Method

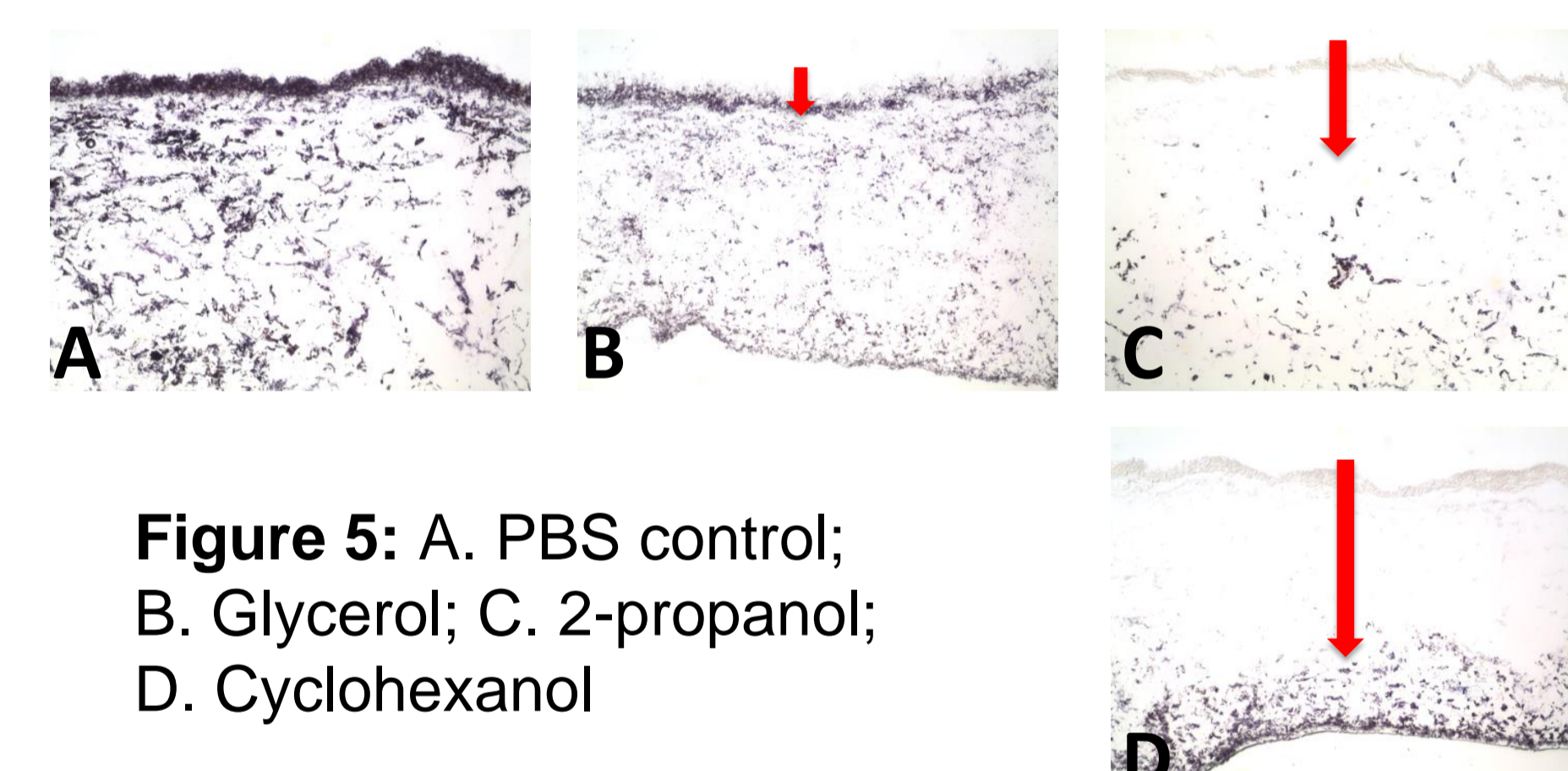
After chemical treatment, tissues were immersed in MTT solution. Then, the tissues were incubated in 20% sucrose and embedded in O.C.T. compound and stored in liquid nitrogen until 14 µm cryo-sections were collected. The depth of injury (DOI) was calculated as % of non viable tissue thickness compared to overall tissue thickness (Fig. 4).

**Figure 4:** Analysis of the cross sections for the quantification of DOI

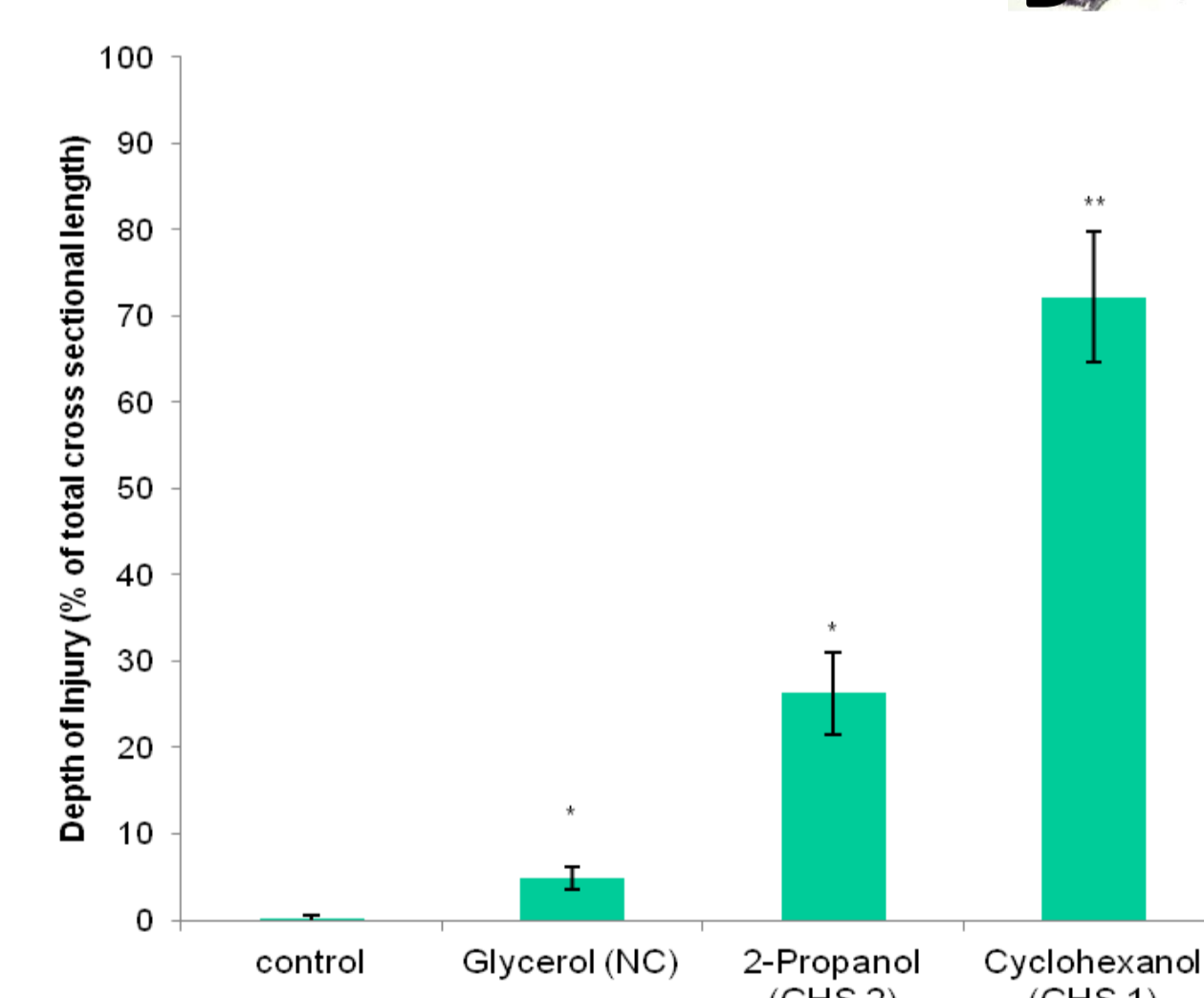


### Results

The tested substances included all GHS categories. Figures 5(A-D) demonstrate the correlation between GHS category and the non-viable tissue fraction, i.e. the DOI, for selected chemicals. Existing results of the DOI and GHS category are summarized in Table 1: GHS no category chemicals result in a DOI < 11%, GHS cat. 2 chemicals affect about 26% of the tissue, and GHS cat. 1 chemicals result in DOI > 70% of the whole tissue.



**Figure 5:** A. PBS control; B. Glycerol; C. 2-propanol; D. Cyclohexanol



**Table 1**

GHS category	Reference chemical	Depth of injury (% of total cross sectional tissue length)
control NC (nonirritant)	PBS	0-0,2
	Dimethyl sulfoxide	11,0
	Glycerol	4,9
GHS2 (mild & moderate irritant)	2-Propanol	26,3
	Ethanol	26,0
GHS1 (severe irritant)	Cyclohexanol	72,2
	10% Triton X-100	99,15

### Outlook

This methods allows the discrimination of all GHS categories. A prediction model can be derived on the basis of this preliminary results.

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References:

[1] Engelke et al. (2013) Tox. In Vitro 27 (1), 458-468

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