Image-based Quantification of Skin Irritation by Spatial Biomarker Profiling

Thora Pommerencke, Kathi Westphal, Claudia Ernst, Hartmut Dickhaus, Niels Grabe

Institute for Medical Biometry and Informatics, University Hospital Heidelberg
Im Neuenheimer Feld 305, 69120 Heidelberg, Germany
thora.pommerencke@bioquant.uni-heidelberg.de

Abstract. We developed a method for the quantification of skin irritation based on the treatment of skin cultures and the subsequent automatic image analysis of fluorescently stained tissue sections being scanned with a fullslide scanner. In this method, the irritative effect is reflected in a shift of the expression pattern of selected biomarkers inside the epithelium. Using the example of SDS as irritant and HSP27 as a marker for skin irritation we showed that our method is capable of quantifying the irritative effect of SDS and furthermore delivering spatial and time dependent information.

1 Introduction

Every chemical or finished product has to be tested for its skin corrosive and irritation potential before it is made available to the human population. The prevailing risk assessment is conducted by means of *in vivo* animal tests. However, scientific concerns about the variability and predictive capacities of animal tests for humans and especially recent ethical reasons led to substantial efforts to develop alternative *in vitro* test methods. The commonly applied alternative method is the MTT test using organotypic culture systems, where the cell vitality is measured inside a homogenate [1]. This is suitable for testing skin corrosion, but not for mild irritation, as the latter does not lead to tissue destruction. In literature, these weak effects are often described qualitatively based on the visual inspection of histological stainings. We now developed a method for the quantification of skin irritation based on the automatic image analysis of intact, fluorescently stained tissue sections from treated skin cultures being scanned with a fullslide scanner. The irritative effect is reflected in a shift of the expression pattern of selected biomarkers inside the epithelium. We here used as irritant the detergent sodium lauryl sulphate (SDS) and the heat shock protein HSP27 [2] as a marker for skin irritation.

2 Methods

2.1 Experimental Setup

Mattek EFT 400 cultures were treated either with nothing, PBS or 0.4 % SDS. For this, 25 μl of the respective solution was applied on top of the cultures on a mesh for different durations i.e. 1 h, 6 h, 16 h and 24 h. Afterwards, the cultures were cryo conserved and cut into sections of 6 μm. For each mode of treatment (e.g. 6 h SDS) there were four cultures and for each culture about 5 sections. The sections underwent an immunhistological fluorescent triple staining, labeling the cell nuclei with the nuclear dye DAPI in blue, the basal lamina using an antibody against the membrane protein Laminin 5 in green (Alexa 488) and the biomarker (i.e. Hsp27) for the quantification of skin irritation was marked in red (Alexa 594). The challenge was here to find a reference staining for the automatic segmentation of the epithelium which is stable against the SDS treatment. DAPI and Laminin proofed to be appropriate.

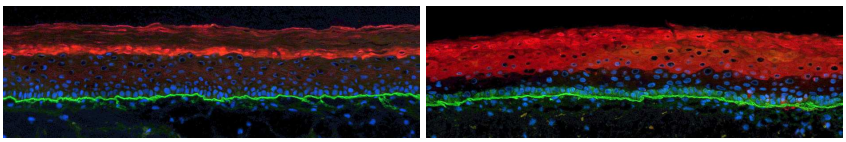
2.2 Image Acquisition

The fluorescent sections were scanned with the Nanozoomer HT from Hamamatsu Photonics at 20× magnification (a resolution of 0.46 μm/pixel). The images were recorded in three z-layers with a spacing of 2 μm each. The resulting slide scans were in a proprietary image format (.ndpi) from Hamamatsu. For further analysis those image regions to be analysed were selected in a computer assisted way and converted to be available for later image processing algorithms.

2.3 Segmentation of the Epithelium

As there was no staining marking the whole epithelium, the epithelial area had to be approximated using the staining of the basal lamina and the cell nuclei. However, having artificial skin cultures one can presume that the epithelium shows almost the same thickness (distance from basal lamina to surface) over the whole section neglecting border regions.

The first step was the segmentation of the basal lamina, visible in the green channel. After contrast enhancement and noise reduction mainly a thresholding based on the Otsu's method is applied. Since sometimes there was an unspecific



(a) 24 h None (b) 24 h SDS

Fig. 1. Immunhistological fluorescent triple staining. Changed Hsp27 expression pattern following SDS treatment.

and diffuse staining in the stratum granulosum and corneum, the segmentation process is supported by an edge detection based on the Canny method parameterized to detect long stretched edges.

The cell nuclei were detected in the blue image channel by a two-step thresholding process [3], where only image areas close to strong image gradients were considered. Cell clusters were identified by size and split using a watershed method with local maxima as seeds. Finally, false positive cell nuclei were filtered out on the basis of object features like size, roundness and contrast. Ring-like stained nuclei, identified by the ratio of border intensity and inner intensity, were treated separately.

For the approximation of the surface line the basal lamina line was smoothed strongly and shifted towards the epithelium by 1.4 times the maximum (95 % quantile) nuclei distance from basal lamina. The shifting direction was determined by the nuclei density in a band around the basal lamina. The epithelial side exhibits a higher nuclei density than the side of the culture's collagen matrix.

To yield in a closed epithelial area with sides vertical to the surface, the surface pixels were fitted with a line and the endpoints of the surface were connected to the intersection points of the perpendicular through the respective endpoint and a point on the basal line.

2.4 Biomarker Profiling

For the biomarker profile the mean intensity in the red image channel was measured inside bands (given by distance intervals) of the determined epithelial area. The determined marker intensity was plotted against the corresponding "relative distance" [4] of the epithelial band. All profiles from one mode of treatment (e.g. 6h SDS) were averaged.

2.5 Feature Selection for the Quantification of Skin Irritation

In case of the protein Hsp27 SDS treatment leads to a premature and cytoplasmatic expression pattern with planar appearance (Fig. 1). In the profile a premature expression pattern is reflected by an early increase of the marker intensity.

To determine this starting point of expression, the section of the profile with the highest slope was fitted with a line. The intersection point of that line with the horizontal through the minimum marker intensity was set as the starting point of expression (*StartExpression*, Fig. 2a).

The planar histological expression pattern is reflected in a high integral of the profile from starting point of expression to profile's end. This feature *ProfileExpressionArea* was calculated by summing up all marker intensities from starting point of expression (Fig. 2b).

As the main change in expression can be observed between 40 % and 70 % relative distance also the sum of marker intensities between 40 % and 70 % relative distance was calculated (*ProfileExpressionArea40-70*). Furthermore, literature

[2] reports a translocation of Hsp27 into nuclei following SDS treatment. For this reason also the mean marker intensity located in the nuclei was measured in the area between 40 % and 70 % relative distance (*NucleiIntensity40-70*). All algorithms described above were implemented in Matlab R2008b.

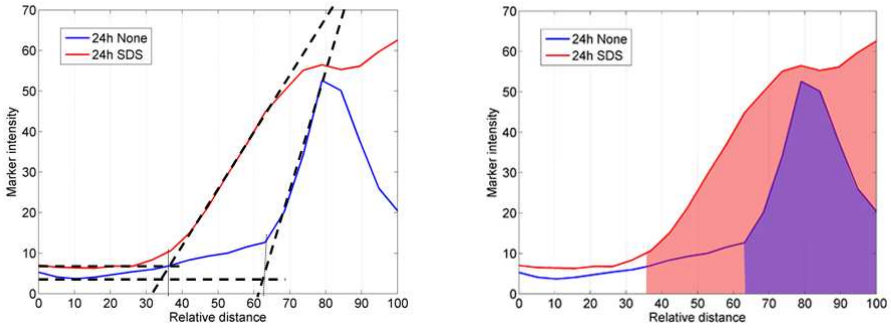
3 Results

In Fig. 3 the median profiles with their 25 % and 75 % quantiles of two modes of treatment (24 h None and 24 h SDS) are shown exemplarily.

Setting the values of the extracted SDS features into relation with the corresponding None values, the SDS results show strong changes in all of the four features, strongest in ProfileExpressionArea40-70. At a treatment duration of 24 h the SDS value increases to a 2.6-fold of the corresponding None value. However, also a treatment of the cultures with PBS leads to a 1.5-fold increase in ProfileExpresssionArea40-70. This means that even PBS has a somewhat irritative effect, possibly due to occlusive effects. Therefore, to measure the irritative effect of SDS, the SDS values are set into relation to the PBS values (Fig. 3c). At a treatment duration of 24 h a 1.7-fold increase in ProfileExpressionArea40-70 is detected.

4 Discussion

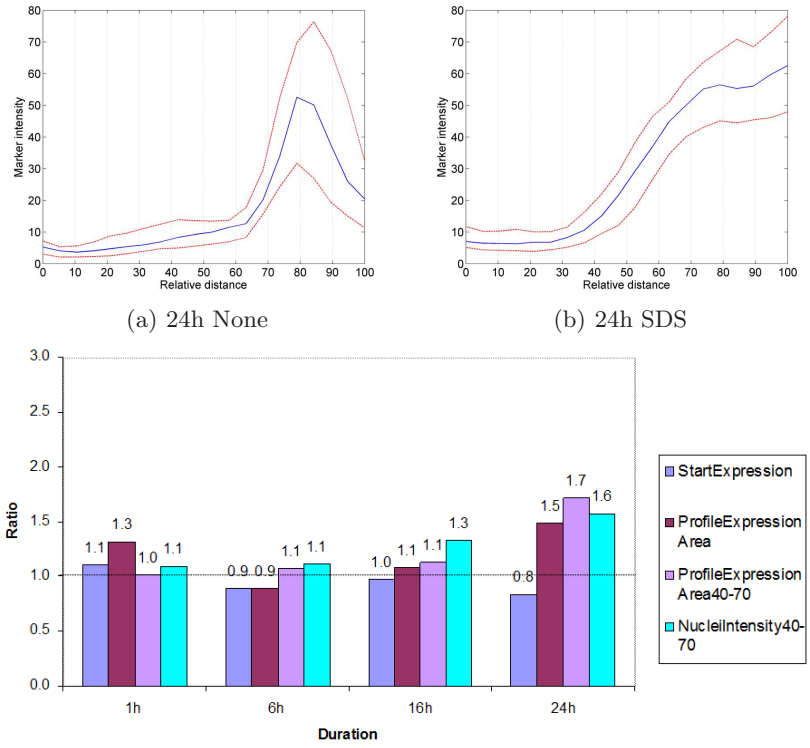
We have developed an automatic method for the quantification and spatio-temporal description of the effect of skin irritation inside the epithelium. Even though the tissue is morphologically still quite intact (not corroded yet), the irritative effect of SDS and even PBS can be measured within a good confidence interval (Fig. 3(a, b)). Complemented by further biomarkers this system could give important insights into the mechanisms of skin irritation. Having also measured an effect at PBS treatment makes this method promising for the test of mildly irritating substances.



(a) StartExpression (b) ProfileExpressionArea

Fig. 2. Feature extraction from the marker profiles.

Fig. 3. Exemplary marker profiles with confidence interval (a,b). Time resolved change in expression after SDS treatment compared to PBS (c).



(a) 24h None (b) 24h SDS

(c) Ratio of SDS and PBS

In future, further reagents like those mildly irritating (e.g. CTAB) have to be tested. To optimize the automatic segmentation, efforts should be put on establishing other histological reference markers allowing for a segmentation, which is independent of epithelial thickness.

References

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