Automated Detection and Analysis of Fluorescent Biomarkers in Human Placental Chorionic Tissue

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Introduction

Functional analysis of placental biomolecules that are involved in pregnancy-supporting processes at the materno-fetal interface requires their *in-situ* allocation to the cellular compartments of the placental chorionic villi (PCV) such as the syncytiotrophoblast (STB). In addition, measurements of qualitative and quantitative alterations in protein expression due to different genotypes and environmental exposures can help to understand diseases such as preeclampsia.

In this context, multimolecular analysis of the PCV compartments in combination with exhaustive bioinformatic knowledge extraction (cytomics) is of growing interest. Cytomics-technologies are often microscope-based using automated *in-situ* identification of cellular systems (tissue areas, cells, subcellular structures) and linear quantification of associated marker molecules (e.g. TissueFAXS/TissueGnostics GmbH, Vienna, Austria). Currently available software, however, does not support the *in-situ* analysis of PCV with complex shapes/textures of villi, multinuclear cells (e.g. STB) or highly autofluorescent nuclear-free erythrocytes.

The receptor for advanced glycated end-products (RAGE) is a multiligand receptor that can elicit inflammatory states, plays a role in development of diseases such as diabetes mellitus, and might also contribute to the development of preeclampsia (PE).

Aim

We aim to push forward the automated analysis of large and complex tissues like the PCV. Acquisitions have to be considered as a whole instead of dealing with single field of views (FOVs), otherwise information about large structures is lost at each border. Furthermore, classical cell-recognition approaches that rely on nuclei staining fail to identify multinuclear cell types such as the STB or highly autofluorescent nuclear-free erythrocytes. Our aim is to develop new approaches for general recognition of such structures called holistic pattern-recognition.

As a specific application for this approach, allocation and level of expression of RAGE in PCV in PE and age-matched healthy (CO) placentas is currently subjected to automated analysis to study alterations of RAGE expression during PE.

Methods

Paraffin-sections of placentas were incubated with primary and respective fluorescence-conjugated (Alexa Fluor 488 and 546, Invitrogen) secondary antibodies (Abs). Nuclei were labelled with DAPI (Roche). Images (individual fluorescent channels, transmitted light) were recorded by automated slide-scan using the TissueFAXS (TissueGnostics GmbH; 20x objective). Pictures (9x9 FOVs) were sampled with an overlap. All adjacent overlaps were used to merge the respective images by using template-matching function of OpenCV. Automated recognition of tissue structures and cells was developed combining classical digital image processing with pattern recognition approaches as well as state of the art machine-learning techniques.

References

[1] Heindl et al. 2010, „Towards a Versatile Automated Cell-Detection System for Science and Diagnostics“, Accepted Paper for 32nd Annual International Conference of IEEE Engineering in Medicine and Biology Society

[2] Viola and Jones, „Rapid object detection using boosted cascade of simple features“, Computer Vision and Pattern Recognition, 2001

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Results

1. STB detection

Figure 1 shows an acquired overlay image composed of all recorded fluorescence channels. The STB is marked in green (Alexa Fluor 488) using a primary anti-cytokeratin 7 (CK7) Ab, while RAGE is marked in red (Alexa 546) using an ectodomain specific primary Ab. All nuclei are stained in blue (DAPI). Two independent domain experts used all stitched channel images to manually mark up the region covered by the STB. An overlay of such a markup can be seen in Figure 2. Green represents the intersection of both experts (agreement of 92%), whereas blue and red show the areas that were only marked up by one of the experts. Using the specific staining for CK7 (Figure 3), we computed a multinuclear cell mask (Figure 4) and achieved an agreement of about 90% with both human experts after exhaustive parameter optimization [1].

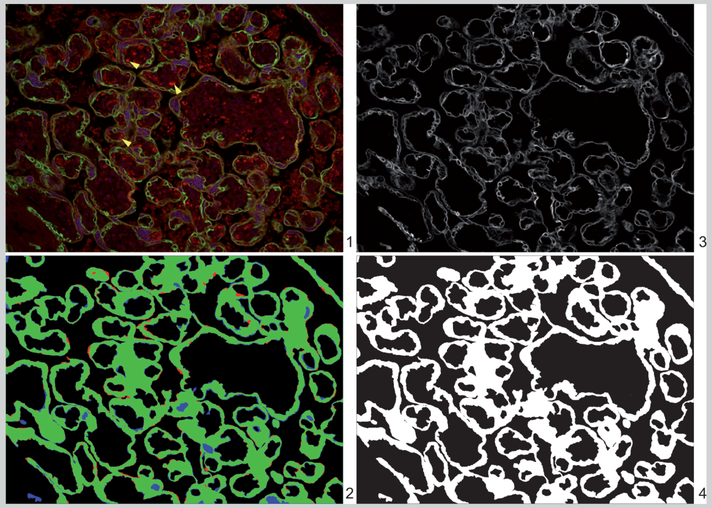

Figure 1-4

These figures show the same FOV of a placental paraffin-section stained with anti-CK7 antibody to detect the STB (green), DAPI to detect nuclei (blue) and anti-RAGE antibody (red). Figure 1 represents an overlay image of all acquired fluorescence channels. The arrowheads indicate spots with significant background fluorescence due to erythrocytes. Figure 2 is a composition of the markups done by our human experts. Green shows the agreement of both domain experts where as red and blue represent the areas where only one of them marked up the region covered by the STB. The STB specific staining is shown in Figure 3. The final mask computed by using our system can be seen in Figure 4.

2. Erythrocyte detection

Significant background fluorescence is generated by erythrocytes in the placenta (see arrowheads in Figure 1) and many other tissues. Later quantifications of proteins of interest can be influenced by that noise. Therefore, we sought to eliminate their associated fluorescence by generating an erythrocyte mask (Figure 7). Classical image processing techniques will fail because there is no single channel, where erythrocytes are solely visible.

To overcome this problem, we made use of the specific shape of erythrocytes in transmitted light (see arrowheads in Figure 5) and used a classifier based on “haar-like features” [2]. These features are commonly used for face detection, but we trained them to detect erythrocytes in transmission images. The output of this classifier is a set of regions that most likely contain erythrocyte (Figure 6). Erythrocyte areas in these regions are then identified by classical pattern-recognition approaches and fluorescent pixels associated with these areas are excluded from quantification of proteins of interest. A corresponding erythrocyte mask to Figure 1 is shown in Figure 7 (insert in Figure 6).

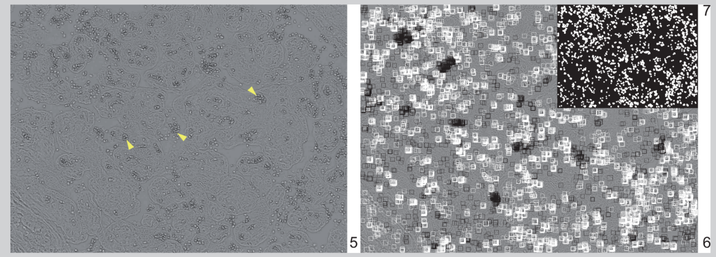

Figure 5-7

Figure 5 shows a transmitted-light image of the same FOV as shown in Figure 1, where arrowheads mark some spots where erythrocytes can be found. The corresponding output of the classifier is shown in Figure 6, highlighting regions containing erythrocytes. The respective erythrocytes mask can be seen in Figure 7.

3. RAGE quantification

As an example for automated allocation and quantification of a placental protein, RAGE expression in 13 PE placentas (age 26+5 to 36+1) and control placentas (23+2 to 36+6) is currently investigated. To study expression, anti-RAGE antibody SC-5563 is used. This antibody detects mainly a soluble form of the receptor (sRAGE; 45-48 kDa) in human placental tissue (Figure 8). RAGE detected by SC-5563 localizes mainly to the STB layer of PCV (red arrowheads), but is also found in cells in the stromal core of PCVs (blue arrowheads, Figure 9).

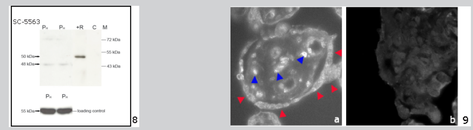

Figure 8-9

Figure 8: Immunoblot using SC-5563 to detect RAGE in total placental lysates (PH), lysate of RAGE-transfected 293T cells (+R) and 293T cells (C). Figure 9: (a) Localization of RAGE in term PCV. Paraffin sections were immunostained with anti-RAGE SC-5563 and a secondary Alexa-568-conjugated antibody and analyzed by fluorescence microscopy. (b) Control incubation without primary antibody were analyzed with the same microscope settings. RAGE localizes to the STB (red arrowheads) and cells in the villous stroma (blue arrowheads).

We have have analyzed approx. 80 FOV/placenta. We can monitor a trend showing that in PE placentas the total amount of RAGE associated with PCVs after subtraction of erythrocyte-associated fluorescence is increased compared to the control (Figure 10). The automated evaluation of allocation (associated with STB or cells within the stromal core) suggests that in PE placentas, RAGE is more expressed in the STB (Figure 11).

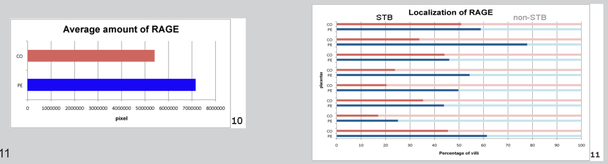

Figure 10-11

The total amount of RAGE proteins quantified in PE and CO placentas is shown in Figure 10, localization within the villi (n=8) in Figure 11.

Conclusion

We pushed forward smart *in-situ* cell analysis by establishing an automated recognition protocol for the multinuceated STB and erythrocytes in PCV sections. Furthermore, we exemplarily quantified a protein of interest, RAGE, in PCV derived from healthy and preeclamptic pregnancies. Current results indicate alteration of RAGE protein expression and allocation in case of preeclampsia, suggesting involvement of RAGE in the development of the disease.