Automated cell-detection technologies for science and diagnostics

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Introduction

Microscopy is a key technique to understand the molecular architecture and functionality of healthy biological systems (e.g. organs) at the single cell level. Alterations in protein expression-patterns caused by diseases can be restricted to small cell populations that might escape detection by molecular or biochemical analysis of whole tissue preparations. However, to study localization and interaction of biomolecules in situ, adequate sample numbers must be investigated and linear quantification of multiple parameters must become possible in an observer-independent and time-saving manner. In this context, automated microscopy technologies (e.g. TissueFAXS/TissueGnostics GmbH) already allow high throughput screens of multiple and large tissue samples. Subsequently applied automated cell-detection technologies aiming for computer-based cell recognition in a tissue-context as well as for observer-independent, quantitative and reproducible quantification of cell-associated markers/parameters are of growing importance in research and diagnostics (e.g. TissueQuest/TissueGnostics). However, automated in-situ identification of special cellular shapes (e.g. multinucler cells) or recognition of large interconnected tissue structures has not been assessed throughly.

The placental chorionic villi (PCV) represent the functional units of the human placenta, exhibt large differences in size and contain a variety of cell types., e.g. the syncytiotrophoblast (STB) that is one multinuclear cell facing the maternal circulation. A plethora of villous proteins that function to guarantee a succesfull pregnancy still awaits in situ studies on localization and interaction with other biomolecules. Furthermore, alterations of localization within the villi and altered expression levels of villous proteins may represent important markers for diseases.[1]

Aims

To improve qualitative and quantitative in situ studies of placental protein expression, we aim to develop a versatile system for holistic pattern-recognition based on human-like interpretations that shall be capable of

i/ Automated recognition of large (> 1 field of view; FOV), interconnected tissue structures (e.g. placental chorionic villi / PCV)
ii/ Automated recognition of multinuclear cells (STB)
iii/ Automated reduction of background fluorescence by erythrocyte detection and subsequent elimination
iv/ Pixel quantification of a(ny) protein of interest/area (e.g. Receptor for advanced glycated end products / RAGE, see Poster 162)

Methods

Paraffin-embedded healthy term placentas were sectioned (4µm) and antigen retrieval was performed. Unspecific binding sites were blocked and sections incubated with primary (see Figure legend) and their respective fluorescence-conjugated (Alexa Fluor-488 and 546, Invitrogen) secondary antibodies. Nuclei were labelled with DAPI (Roche) and samples embedded in Fluoromount GTM (SouthernBiotech). Images (individual fluorescent channels, transmitted light) were recorded by automated slide-scan using the TissueFAXS (TissueGnostics; 20x objective). Pictures were sampled with an overlap. All adjacent overlaps were used to merge the respective images by using template-matching function of Open-CV. The images were acquired and stored without compression in PNG. Automated recognition of tissue structures and cells was developed combining classical digital image-processing and pattern recognition approaches.

Results

1. Stiching

Important prerequisite for computer-based recognition of areas larger than one FOV (e.g chorionic villi) was an automated image stitching process that aligned images at subpixel resolution. Following automatic acquisition, a multiplicity of captured pictures was routinely stitched to create one picture of the entire tissue area selected. Subsequent operations were performed on this „stiched“ image.

2. Villi detection and detection of multinuclear cells

Villi detection was done by intelligent thresholding. Fig. 1 shows PCV, where the surface cell layer (STB) is marked in green (using anti-CK7) and all nuclei are stained in blue. First, a mask that distinguished between background and foreground was created. Fig. 2 shows such a mask marking villis as white and background as black. Note that not all villis are well seperated at this point. To recognize even touching villis correctly, we took advantage of specific characteristics of the multinuclear STB (e.g. expression of cytokeratin 7 (CK7) and a ribbon-like alignment of nuclei). We calculated a mask containing the STB of all villi, representing the **multinuclear cell mask** (Fig. 3). This mask was subtracted from the **background mask** resulting in our final **villi mask** (Fig. 4) used in further quantification of placenta-associated proteins (e.g. RAGE).

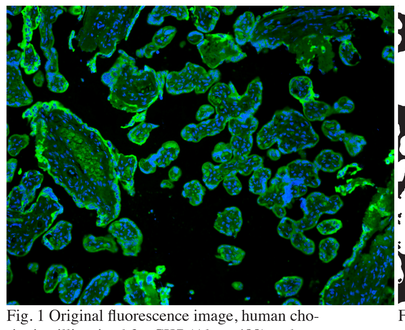

Fig. 1 Original fluorescence image, human chorionic villi stained for CK7 (Alexa 488) and DAPI (overlay) (1392x1024 pixel)

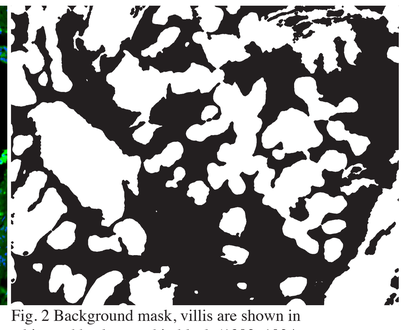

Fig. 2 Background mask, villis are shown in white and background in black (1392x1024 pixel)

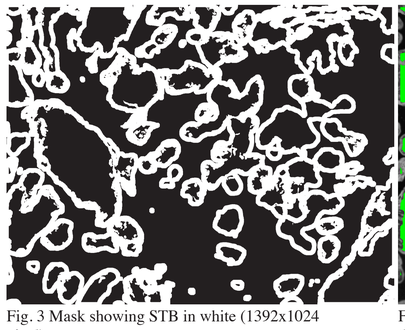

Fig. 3 Mask showing STB in white (1392x1024 pixel)

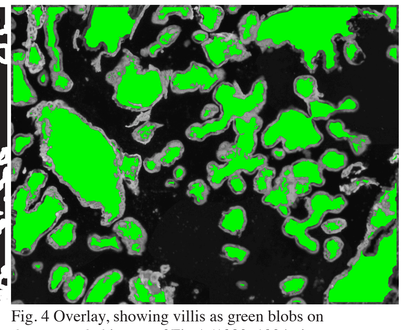

Fig. 4 Overlay, showing villis as green blobs on the grayscaled image of Fig.1 (1392x1024 pixel)

3. Erythrocyte detection

In immunofluorescence microscopy, erythrocytes can contribute significantly to background fluorescence (Fig. 5). We seeked to eliminate their associated fluorescence by generation of an erythrocyte mask. Fortunately, erythrocytes have a very specific shape and strong edges that allows for their separation. Fig.6 shows a transmission image of placental tissue containing erythrocytes. We used various techniques to detect the erythrocytes. Via an intelligent threshold we got the darkest most visible erythrocytes. Additionally, canny edge-detector was applied which found also the brighter erythrocytes. Finally, we moved a 9x9 pixel window over the whole image and calculate standard deviation of the grey levels. The resulting erythrocyte mask can be seen in Fig. 7. Pixels associated with this mask were subtracted from every image.

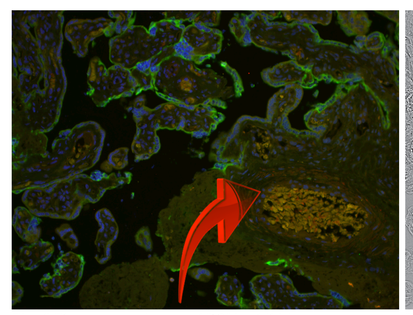

Fig. 5 Fluorescence image of PCV exhibiting background due to erythrocytes (1392x1024 pixel)

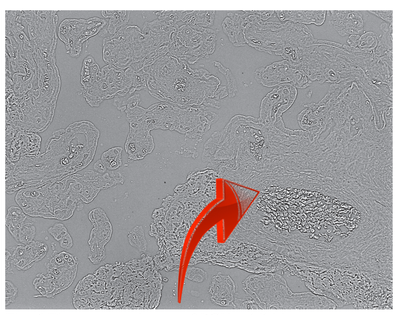

Fig. 6 Fluorescence image of PCV exhibiting background due to erythrocytes (1392x1024 pixel)

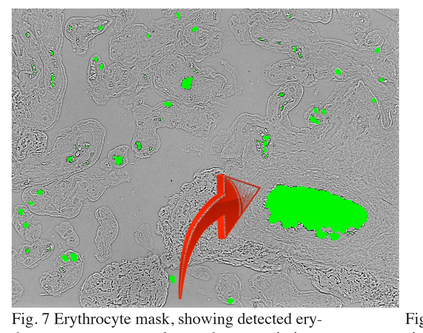

Fig. 7 Erythrocyte mask, showing detected erythrocytes as green overlay on the transmission image of Fig. 6. (1392x1024 pixel)

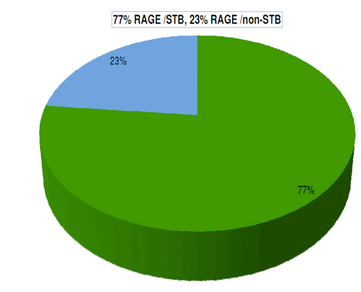

Fig. 8 Quantification of RAGE protein expression in PCV (10544x6112 pixel)

4. Protein (RAGE) quantification

As an example for automated localization/quantification of placental proteins, the expression of RAGE (receptor for advanced glycated end-products, see poster 162) was analyzed. Placental sections were stained for RAGE (Alexa 546), CK7 (Alexa 488) and nuclei (DAPI) and pictures were automatically sampled. Villi mask and multinuclear mask and their corresponding areas were calculated. Fluorescence associated with erythrocytes was subtracted. Quantification of RAGE-fluorescence was done by determining a threshold in negative-control images (no primary antibodies) and thresholding the target images with that value. By using the villi and multinuclear cell mask, localisation of the protein was determined and the amount of fluorescence associated with a certain area calculated (Fig. 8).

Summary

We established an automated recognition protocol for PCV and the multinucleated STB as well as contaminating erythrocytes that is applicable on placental sections stained by standard immunofluorescence techniques to localize and quantitate fluorescence associated with a protein of interest.

Future aims are the discrimination of stem villi and terminal villi as well as the development of an endothelial cell mask.

References

[1] From quantitative microscopy to automated image understanding, K. Huang and R.F. Murphy, Journal of Biomedical Opics 9(5), 893-912

Acknowledgements

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