Proposals for best-quality immunohistochemical staining of paraffin-embedded brain tissue slides in forensics

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Received: 17 October 2017 / Accepted: 15 December 2017
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Abstract
Immunohistochemistry (IHC) has become an integral part in forensic histopathology over the last decades. However, the underlying methods for IHC vary greatly depending on the institution, creating a lack of comparability. The aim of this study was to assess the optimal approach for different technical aspects of IHC, in order to improve and standardize this procedure. Therefore, qualitative results from manual and automatic IHC staining of brain samples were compared, as well as potential differences in suitability of common IHC glass slides. Further, possibilities of image digitalization and connected issues were investigated. In our study, automatic staining showed more consistent staining results, compared to manual staining procedures. Digitalization and digital post-processing facilitated direct analysis and analysis for reproducibility considerably. No differences were found for different commercially available microscopic glass slides regarding suitability of IHC brain researches, but a certain rate of tissue loss should be expected during the staining process.

Keywords Biomarker · Evaluation · Forensic neuropathology · GFAP · Immunohistochemistry · Traumatic brain injury

Introduction
Forensic neuropathology needs biomarkers or proteins with high specificity to structures of the central nervous system (CNS) to better understand and illustrate different pathways activated or lost after brain tissue damage, either following an ischemic/hypoxic or a traumatic event [7, 9, 12, 15]. These results can be used to establish microscopic vitality signs or even to estimate minimal survival times after traumatic impact. Thus, it is necessary to carry out reproducible and reliable studies on human postmortem brain tissue using different methods, especially histological and immunohistochemical (IHC) techniques [5, 8, 10, 11].

In the literature different staining procedures, different counting/interpretation systems and differences in investigated brain regions currently lead to a lack of comparability of the presented results. Furthermore, no standard has been defined for what brain region to use to answer specific forensic questions. This complicates the translation of research findings into daily forensic routine significantly.

Recently, Sabatasso et al. [16] reported about technical improvements of IHC methods used in cases of myocardial ischemia. This excellent report has prompted us to critically review the procedures in forensic neuropathology.

Therefore, the aim of our study was to find a standardized approach to process and analyze IHC in brain tissue slides of forensic issues. This could pave the way for a prospective interinstitutional usage of research data in daily routine, especially to be recognized as proven evidence at a court of law.

We exemplarily used glial fibrillary acidic protein (GFAP), one of the most widely studied proteins in forensic neuropathology. More specifically, we wanted to analyze whether hand-stained or automatic-stained IHC slides of brain tissue lead to more comparable results. We also tried to detect possible differences in suitability between commercial glass slides and to discuss several ways for digitalization of the data.
Materials and methods

Pretreatment and tissue processing

Directly following brain removal during autopsy, fresh brain tissue samples from predefined areas were collected (local regulation: at least a two-sided sample of cortex, the hippocampal formation and the cerebellum; in cases with traumatic CNS damages more samples are taken or the brain in total is stored for a full neuropathological evaluation). The tissue was then fixed in 4% neutral buffered formalin. The brain samples were stored in small cubes of about 1 cm³ to rest for at least 1 week for complete fixation. To avoid impurities during fixation the formalin solutions got changed initially after 24-h once.

The formalin-fixed samples were then embedded in paraffin and 3-μm-thin sections were prepared and mounted on microscope glass slides, then dried at room temperature (20–25 °C) for approximately 12 h.

All tissue sections were first stained using hematoxylin and eosin (H&E) for morphological evaluation of different cell types and detection of inflammatory, ischemic, traumatic or neoplastic changes.

Comparison of microscope slides

In our previous research trials as well as in daily routine, we repeatedly observed a partial or even complete peeling off of the mounted sections from glass slides during the staining process. To detect the underlying phenomenon, we discussed potential reasons. Beside fixation time, thickness of the tissue sections or the staining process itself, possible differences in quality of microscope glass slides were one attempted explanation. To rule out this fundamental uncertainty, we compared results on two commonly set-in microscope glass slides, which were available in both of our laboratories, in detail Superfrost™ PLUS slides (Thermo Fisher Scientific Inc., Waltham, USA) and IHC Microscope Slides FLEX (DAKO Agilent Technologies, Santa Clara, USA).

Therefore, a total number of 100 brain slides with GFAP immunostaining were selected in a randomized way, 50 samples stained on Superfrost™ PLUS and 50 samples on IHC Microscope Slides FLEX. Each of these two test groups was further divided in two subgroups in which 25 samples got stained by hand and 25 automatically (for different staining specifications see paragraph Staining procedures below).

Overall, three stages of the peeling off effect were clearly differentiable: a completely preserved section (see Fig. 1a); a partial peeling off with a loss of segments <20% of the entire section, where evaluation of all relevant structures was still possible (see Fig. 1b), and lastly a complete loss of histological relevant structures making sufficient evaluation impossible (see Fig. 1c).

Of course, these kinds of destructions may have also been caused by the cutting process itself. We used a Leica sliding microtome SM 2000R (Leica Biosystems, Nussloch, Germany) and mounted only complete and un-harmed sections on glass slides to rule out influences on the peeling off rate.

The analyses of the results concerning the peeling off effect are presented in Table 1.

Staining procedures

For both procedures we used polyclonal rabbit anti-GFAP-antibody as primary antibody, isolated from cow spinal cord (Dako) which reacts strongly with human GFAP according to the manufacturer details. The antibody required pretreatment by microwave for 20 min, in citrate buffer at pH 6.0. To block the endogenous peroxidase, the sections were immersed for 10 min in peroxidase blocking solution (Dako).

The primary antibody was applied at a dilution of 1:3000 for 30 min at room temperature. To verify the findings and to confirm antibody specificity, positive and negative controls were prepared consequently for every staining charge in our researches.

Manual staining

Manual IHC staining was performed using the labeled streptavidin-biotin method with specific primary antibody (see above), a biotinylated secondary antibody as well as a streptavidin-complex with labeling. The staining process was completed with a chromogen (3,3′-diaminobenzidine) in hydrogen peroxide/phosphate-buffered saline and counterstaining was performed with hematoxylin (Mayer’s).

Automatic staining by Benchmark XT (Roche)

For automatic staining, we used the fully automated immunostainer Benchmark XT (Roche, Basel, Switzerland). Staining protocol included deparaffinization and counterstain with hematoxylin and blueing reagent according to manufacturers’ instructions. Pretreatment of slides was not necessary for GFAP staining.

Image digitalization

Research in forensic neuropathology investigates the number, form, and/ or intensity of stained cells and cell structures. Traditionally, the most widely used method is to inspect and count structures directly through the ocular lenses. In order to increase reproducibility and reliability, it is recommended to digitalize microscopic images.

For the digitalization, the authors used a Zeiss Axioskop 40 (Carl Zeiss AG, Oberkochen, Germany)
in combination with a camera system *Olympus DP22* with matching adapter *TV 2/3" and 0.63× internal optical magnification* (Olympus K.K., Shinjuku, Japan). Normally, preferably standardized data needs to be counted in high-power fields (HPF), commonly defined as ×400 magnification.

In this process, it was noticed that the produced digital image on screen at microscope settings for ×400 magnification (standard 10× ocular lenses and 40× objective lens) was just a fraction of the image that could be observed through the ocular lenses. After some investigations and optical calculations, which are presented below in mathematical description in detail (see Table 2) as well as illustrated (see Fig. 2), we discovered that a digital image which represents 91% of a common HPF at ×400 optical magnification can be created digitally by using a ×20 objective lens with the described hardware.

### Counting methods

One option of counting relevant structures is the by-eye scanning of slides directly through the ocular lenses. The digitalization of optical images opens up more possibilities in analyzing samples. Examples of such approaches are using additional software provided by manufacturers of microscopes and camera systems, commercial applications for computers or tablets, or different kinds of image analysis software. However, these are just some of the many options available. In our study, we used a software from *Olympus*, which is part of the camera system *DP22* and the commercial object counting application *SIGMA* (open source; developed by Karen Grigoryan 2016) and can be run on tablets.

Unlike the by-eye scanning method, the software includes the option to save counting marks to the digital images as well as unmarked “initial” data, thus allowing to verify and compare results of every single examiner, retrospectively.

### Table 1  Number of peeled off sections subdivided in used microscopic slide, type of staining, and extent of tissue loss

|                | Manually stained |        | Automatically stained |        | Counted |
|----------------|-----------------|--------|-----------------------|--------|---------|
|                | Partly peeling off | Complete peeling off | Partly peeling off | Complete peeling off |        |
| *Superfrost*+  | 4                | 0      | 3                     | 1      | 50      |
| *Dako IHC*     | 3                | 0      | 6                     | 0      | 50      |
| *Counted*      | 25               | 25     | 25                    | 25     | 100     |
Results and discussion

The authors highly recommend the new technical note by Sabatasso et al. presenting interesting insights in using alternative IHC staining methods for detection of myocardial infarctions [16]. They concluded the outmost importance of suitable laboratory methods for the routine work of forensic pathologists. This was the reason for us to present and discuss some relevant pitfalls in IHC of brain tissue slides just to underpin the necessity of high laboratory standards and quality in forensic research again.

In our study, no relevant differences were detected between the two commercial microscope slides. As it can be seen in Table 1, both products seem to be equally suitable and there seem to be only negligible, if any, effects on the peeling off ratio by usage of different kinds of slides.

A presumed explanation for the little higher rate of tissue loss during automatic staining could be the mechanical treatment of slides, compared to the manual processing.

Our main results indicate, that automat-stained IHC slides of brain tissue lead to a higher comparability for research questions than hand-slides, at least for GFAP.

Although manual staining process showed accurately stained astrocytes in the majority of slides, there are known recurrent failures of which some are depicted in Fig. 3.

In contrast, nearly all investigated automatic stained slides presented more consistent staining even in meticulous astrocytic processes and no unspecific background staining (for exemplarily staining results see Fig. 4).

Until now, GFAP as a well-known astroglial structure protein is known to be expressed more or less time-dependent [5, 11, 12] and indicative for astrocytic damage and/ or reparation in different cerebral pathologies, then called astrogliosis [2, 18]. This can be observed after traumatic brain injuries at least surrounding the primary impact area (pericontusional zone) and depending on survival times after trauma. A (semi-) quantitative determination of the glial reaction can then be used to estimate the wound age of an injury [6–8]. To make reliable statements concerning this topic, it is essential to have solid reference data and create consistent staining results in every individual case, both in research and in daily routine.

Therefore, even most of the forensic laboratories should be able to get automatically stained GFAP slides, either by own technical equipment or by a sufficient cooperation to the colleagues of clinical (neuro)-pathology. Of course, an experienced laboratory worker is able to get reliable IHC stainings even by hand-techniques, but stainings performed in different laboratories often suffer from a low inter-laboratory comparability. Also, this applies to IHC stainings performed in the same laboratory but at different time points. Hence, there is a high need to develop optimal procedures for sufficient and reliable results. Not without reason automated IHC staining is often said to be a “milestone” in standardization [14].

Since it is known that insufficient or incorrect fixation tend to false positive staining results, it is crucial to optimize this first step of the process chain according to evidence based guidelines [3]. Next to this, careful positive and negative control slides should be included every time for quality assurance reasons.

Of course, both compared staining procedures were carried out with comparable detailed method sheets as “classic” labeled streptavidin-biotin-method. Changing the method

| Table 2 Calculations of displayed size ratio between optical field of view (oFOV) at × 400 magnification and digital FOV (dFOV) at × 20 and × 40 objective lens in connection with adapter TV 2/3° (Olympus). With × 40 objective lens only 23% of an optical high-power field (HPF) is displayed on screen, while 91% of an optical HPF can be presented on screen with the use of × 20 objective lens |
| --- |
| **Calculation field of view [FOV] Zeiss Axioscope 40** |
| (23mm² × eyepiece magnification [em]) ÷ objective magnification [om] = diameter [d] FOV diameter [d] ÷ 2 = radius [r] |
| **Optical FOV at × 40 [oFOV⁴⁰]** (common high-power field) (23mm² × 10 [em]) ÷ × 40 [om] = 0.575 mm [d] 0.575 mm ÷ 2 = 0.288 mm [r] Circle surface [A] = π × r² oFOV⁴⁰ = 3.141 × (0.288 mm)² = 0.260 mm² |
| **Digital FOV at × 40 [dFOV⁴⁰]** Length of display representation² [l] × heigth of display representation² [h] = screen surface [A] 0.281 mm [l] × 0.211 mm [h] = 0.059 mm² [A] |
| **Digital FOV at × 20 [dFOV²⁰]** Length of display representation² [l] × heigth of display representation² [h] = screen surface [A] 0.561 mm [l] × 0.421 mm [h] = 0.237 mm² [A] |
| **Ratio between dFOV⁴⁰ and oFOV⁴⁰** 0.059 mm² ÷ 0.260 mm² = 0.227 0.237 mm² ÷ 0.260 mm² = 0.912 **Ratio between dFOV²⁰ and oFOV⁴⁰** |

1 Mechanical information given by Olympus 
2 Measured with digital ruler tool on screen
details to other techniques like peroxidase-anti-peroxidase or dextran-polymer-method might present both advantages and disadvantages. Examples could be the suppression of the ubiquitous occurrence of biotin and the affection of this substance on background staining, higher sensitivity, and different consistency in staining results [10, 16, 17] but this aspect was not part of the presented report. We also want to point out that manual staining is still indispensable. New or rarely used antibodies are applied in general by hand and every step in the staining procedure can be adjusted much more flexible compared to automatic staining [3]. But for the special requirements to reliability and reproducibility concerning evaluating brain IHC, automatic staining seems to be more suitable in forensic pathology. If IHC results should be used in forensic reports as evidence, high quality demands should be added to such expert statements to avoid misdiagnosis or overinterpretation of data because counter-assessments gets more and more popular in jury trials and inaccurate appraisals may cause lasting legal consequences of the defendant.

Overlooking the relevant literature of IHC brain investigation, it seems necessary to point out that in many publications, it is indistinct whether the authors counted structures at optical magnification or digital illustration with original or adapted magnification. This hampers comparability across different research groups. In our point of view, it is essential to give more conclusive information about the counting area in detail and create standardized and comparable data. Own efforts with adaptation of the investigated magnification to square millimeters were laboriously programmed but failed to be transferable from research in daily routine [1].

To increase the reproducibility of counting data, it is recommended to digitalize microscopic images, which allows all internal and also external examiners to use the exact same sectors from the sample for re-evaluation. Concerning digitalization of histological prepared tissues, the technique of whole slide scan represents the highest technical development stage currently, because it enables a real digital pathology [13]. For financial reasons, a widespread usage of this technology is not practicable for many forensic institutes at the moment but will become more and more popular in the future. This development (“all-digital”) will provide unprecedented possibilities of extensive consultation and professional exchange in real time all over the world just using digital platforms. For today, the creation of single digital images represents an adequate solution in our opinion, particularly as these data could be utilized in different ways, for example for the evaluation with image analysis software. Not least, providing the exact same digital images from predefined areas to two independent investigators disables most of the interobserver issues from scratch, which seems to be the biggest problem in human-based assessment of IHC stained samples [4]. The additional application of any software solution could increase reliability and comparability of outcomes - both intra- and interinstitutional - noticeably.

The given report has some limitations. As always, forensic IHC research as one kind of semi-quantitative

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**Fig. 2** Visualization of size ratio between an optical high power field (HPF) seen in ocular lenses (white circle; a × 10 magnification ocular lenses + × 40 objective lens), customized setting for digital camera displayed close-to-HPF image (outer red square; b adapter magnification × 0.63 + × 20 objective lens magnification) and digital camera image with original setting for optical HPF (inner red square; c × 0.63 + × 40 objective lens magnification)

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**Fig. 3** a–c Typical results from manual staining of GFAP-immunohistochemistry, magnification × 200. Background staining and fluctuation in staining intensity could be observed more often
assessment misses strict “black-white” differentiations. As well as there may be perfect hand-stained slides, there will be a few unusable automatic stained ones. The authors are also aware of alternative error sources for the peeling off effect, mentioned as time of fixation, thickness of mounted sections or used dilution of primary antibody. These parameters had already been optimized through our own past studies regarding decent staining results [1, 5, 10, 18]. Therefore, we currently drew the attention on the questionable suitability between different microscopic glass slides.

Furthermore, the underlying sample size is relatively for generalization concerning the peeling off ratio. Nevertheless, the authors argue to work and research as precise as possible and to circumvent known vulnerabilities using laboratory approaches such as IHC.

A formal wish for the future will be to achieve standardization among clinical and forensic IHC laboratories, but this might stay as an ambitious goal. However, the laboratories might benefit from unified quality control checklists for staining procedures [13].

Other future steps to promote consistent quality standards could be the establishing of ring trials as well as regular staff trainings concerning microtome cutting and staining procedures to the forensic staff. This has already been proposed in the USA with additional certificate programs for histotechnologists by the American Society for Clinical Pathology [13].

Conclusions

• Automatic staining can increase the reliability and comparability of the IHC evaluation of brain samples.
• Manual staining is still indispensable for new or rarely used antibodies in both research and daily practice.
• There seems to be no differences in suitability of the tested microscopic glass slides, a certain rate of tissue loss should be expected.
• Digitalization of microscopic images enables numerous ways of reproducible analysis.
• Digital image displaying contains an alteration in image size depending on the used camera systems, which should be taken into account when spreading associated data.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest. Indeed, they did not receive any funding from the named companies. All of them are not aware of this method paper and the presented results.

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Fig. 4 a–c Typical results from automatically staining of GFAP-immunohistochemistry, magnification × 200. Staining is more consistently, background is plainer and negative structures can be distinguished quite easy.
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