From fix to fit into the autopic human brains

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Abstract

Formalin-fixed, paraffin embedded (FFPE) human brain tissues are very often stored in formalin for long time. Formalin fixation reduces immunostaining, and the DNA/RNA extraction from FFPE brain tissue becomes suboptimal. At present, there are different protocols of fixation and several procedures and kits to extract DNA/RNA from paraffin embedding tissue, but a gold standard protocol remains distant. In this study, we analysed four types of fixation systems and compared histo- and immunostaining. Based on our results, we propose a modified method of combined fixation in formalin and formic acid for the autopic adult brain to obtain easy, fast, safe and efficient immunolabelling of long-stored FFPE tissue. In particular, we have achieved an improved preservation of cellular morphology and obtained success in post-mortem immunostaining for NeuN. This nuclear antigen is an important marker for mapping neurons, for example, to evaluate the histopathology of temporal lobe epilepsy or to draw the topography of cardiorespiratory brainstem nuclei in sudden infant death syndrome (SIDS). However, NeuN staining is frequently faint or lost in post-mortem human brain tissues. In addition, we attained Fluoro Jade C staining, a marker of neurodegeneration, and immunofluorescent staining for stem cell antigens in the postnatal human brain, utilizing custom fit fixation procedures.

Introduction

It is often difficult to obtain reproducible immunohistochemical signals and to perform genomic analysis from post-mortem brain tissues, because the elapsed time from the death of the patient, the time of fixation, and the time of embedding may influence the preservation of antigens and nucleic acids.1-4 In fact, researchers frequently prefer to exclude autopic brain tissues from their experimental cases because the quality of nucleic acids varies in different post-mortem samples. However, it would be very interesting to obtain good quality autopic tissue, both to have autopic “normal” controls and to compare the genomic data from surgical and pathological specimens versus autopic brain tissues.

The effect of post-mortem delay and fixation time on immunohistochemistry and immunofluorescence outcome is crucial pre-analytical variables that can influence genomic analysis and immunohistochemistry outcome. A delay on fixation, as well as the time of formalin fixation can reduce the immunostaining efficiency.1-5 Preservation of proteins in relation to the post-mortem delay is sporadically calculated, and, in the routine setting, the post-mortem delay might range from a few hours to several days. Several biochemical changes occur in the cadaver, including a decrease in the pH, which influences the exposure of target proteins and epitopes. However, this detection loss has received little attention from the scientific community.5-7 The usual fixative is 10% formalin, which consists 3.7% formaldehyde in water with 1% methanol. Formaldehyde is a reactive electrophilic substance; it reacts promptly with various functional groups of biological macromolecules in a cross-linking mode.8 Formalin degrades DNA and masks protein epitopes by forming covalent bonds between neighbouring proteins or nucleic acids. The initial cross-linking is formed by 24 to 48 h after penetration while the concluding may take about 30 days to generate the stable covalent cross linkages. The initial phase of the reaction is reversible while the later reaction becomes irreversible when there is high number of covalent bonds produced. This modifies the physicochemical state of tissue such as redox and membrane potentials of the tissue, changing surface charges and therefore alters the reactivity of cellular components. The reversible nature of the initial phase reaction is the basis of antigen retrieval in molecular techniques. To unmask epitopes for antibody binding in formalin-fixed tissue, several enzymatic and heat-induced antigen retrieval methods have been introduced. These methods are not completely successful for all antibodies and not all unmasking procedures are usable for every antibody.6,14-16 Formic acid is generally used to virtually eliminate the risk of handling infectious material of autopic tissues from patients with prion diseases.15 Formic acid pre-treatment may also produce an antigen retrieval effect, enhancing immunostaining.6,16-17

The present study aims at examining the effect of four different types of fixation on the morphological preservation, histostaining and immunoreactivities of several antibodies, against formalin-sensitive antigens, that do not produce optimal result in post-mortem human brain tissue. In other words, we intend to compare formalin fixation, i.e. the standard and more common method, versus the traditional fixative solution mixed with formic acid and/or acetic acid respectively. We chose formic acid for its anti-prionic property and putative unmasking capacity. At the same time, we selected acetic acid, an efficient dehydration agent,
to reduce oedema of cerebral tissues. Clarke (1851) was the inventor of using a combination of alcohol and acetic acid for fixation, yet many authors attribute Carnoy (1867).18,19 Currently, the formalin, alcohol and acetic acid mixed protocol is utilized for foetal brain fixation.20,21 We decided to modify and utilize the foetal protocol for perinatal and postnatal brains, dividing the time of fixation in steps of four days for each passage (Table 1). We adapted protocols for adult brain fixation according to this postnatal protocol, to unify all of protocols in time point of multiple of four days. We chose the final time of formalin fixation in twenty days, according to the time course for both generation of stable covalent cross linkages, global volume shrinkage and formalin-induced changes of myelin.9,22,23 We linkages, global volume shrinkage and formalin-induced changes of myelin. To exclude any variability of antigen expression related to the Brodmann areas considered, we preliminary prepared different cortex blocks, taken from all cortical lobes of the brain fixed in formalin, therefore we compared antigens expression and nucleic acids extraction for each area.

**Human postnatal brain tissue**

Five autopic postnatal brains were obtained from patients of about one month, who died for sudden unexpected infant death (SUID). We included a postnatal brain from a hypoxic ischemic encephalopathy case as our positive control. Again, brain fixation has been considered our internal negative control, because from this tissue we have never obtained positive immunostaining against formalin-sensitive antigens, as it usually happens with post-mortem brain tissues.

We propose a protocol to standardize the NeuN staining of autopic brains by automatic machine staining. Furthermore, we propose to use Fluoro Jade C, a histochemical technique currently used only to detect neurodegeneration in animal models, for human autopic diagnoses.24,25

**Materials and Methods**

**Human adult brain tissue**

The study was approved by the “S. Anna” University Hospital Ethical Committee within the Project for Human White Matter Study of the Ferrara’s University.26 post-mortem cerebral tissue was obtained from five adult patients without any previous history or symptoms of neurological disease. To avoid cross ethical problem related to the precious extraction of the brains, and to reduce the variability of preanalytical phase, all tissues were extracted 40 h post-mortem.3 During autopsy, the fresh brain was cut in coronal macro-sections of 1 cm thickness. In this way, four serial sections included the hippocampus.27 Following post-mortem macro-dissection, adjacent coronal slices were immersed in four different containers according to four different protocols of fixation (four containers for each post-mortem brain). One additional coronal macro-section at the level of the hippocampus was obtained from a patient who died for a non-neurological cause and was affected by monolateral temporal post-traumatic epilepsy. This macro-section was fixed in 10% neutral-buffered formalin for almost three months.

All tissue blocks used in this study were from the temporal lobe and the hippocampus. To exclude any variability of antigen expression related to the Brodmann areas considered, we preliminary prepared different cortex blocks, taken from all cortical lobes of the brain fixed in formalin, therefore we compared antigens expression and nucleic acids extraction for each area.

**Types of fixation**

We used four different methods of fixation for the adult human brain, which we named A, B, C and D type. In the A protocol, i.e. the traditional protocol of post-mortem tissue fixation, we put macro-slices in 10% neutral-buffered formalin for 10 days. In the B protocol, we used 10% neutral-buffered formalin to fix but, at the 9th day, we put the macro-slices in 98% formic acid for 1 h, just before the last passage in ethanol for 1 h, just before processing and paraffin embedding. In both the A and B protocols we changed the formalin after 24 h and then every four days, for a total of five times.

In the C protocol we used a liquid fixative (LF) based on a 10x glacial acetic acid solution in 10% neutral-buffered formalin. The LF was changed after 24 h and then every four days, for a total of four times. In the last four days we moved the slices in 70% ethanol. The D protocol was identical to the C, except that at the 9th and 17th day we incubated slices in formic acid for 1 h, just before the last passage in ethanol for four days (Table 1).

The macro-slice from the epileptic post-traumatic brain was fixed using the A protocol for three months. For postnatal brains, we used the LF described above for protocol C, which was changed every day for 8 days. Thereafter, brains were plunged into 70% ethanol for 4 days, then in fresh 80% ethanol for another 4 days and finally cut in macro-slices to conclude the fixation using a 95% ethanol solution for 4 days (Table 2).

**Paraffin embedding**

To exclude the variability due to postfixation processing, all tissue blocks were processed and embedded in paraffin within 1 week. Paraffin embedded blocks were cut in 7 µm sections for histology and immunohistochemistry, or in 10 µm sections for genomic analysis, using a microtome (Leica, Wetzlar, Germany). Sections were mounted on adhesive microscopy slides (Superfrost plus, Thermo Scientific, Waltham, MA, USA) and assigned for staining and RNA purification and amplification to researchers who were blind of the group to which each section belonged.

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**Table 1. Adult fixation protocols.**

|     | Liquid fixative* | ETOH 70X | ETOH 80X | Cutting in | ETOH 95X
|-----|------------------|----------|----------|-----------|----------|
| A   | 1 day + 5 x 4 days in formaline (21 days) |          |          |           |          |
| B   | 1 day + 2 x 4 days (9 days) in formaline + formic acid 1 h + 3 x 4 days (12 days) in formaline + formic acid 1 h (before processing) |          |          |           |          |
| C   | 1 day + 4 x 4 days (17 days) in LF (Formaline + acetic acid) + ETOH 70X 4 days |          |          |           |          |
| D   | 1 day + 2 x 4 days (9 days) in LF + formic acid 1 h + 2 x 4 days in LF + formic acid 1 h + ETOH 70X 4 days |          |          |           |          |

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**Table 2. Perinatal fixation protocol.**

| Liquid fixative* | ETOH 70X | ETOH 80X | Cutting in | ETOH 95X
|------------------|----------|----------|-----------|----------|
| 8 days in liquid fixative | 4 days | 4 days |           |          |
| 4 days (changed each day) |          |          |           |          |

*Glacial acetic acid (10x solution) in 10% neutral-buffered formalin
Histology and immunohistochemistry

**Automated staining and immunostaining**

The haematoxylin and eosin (H&E) stain was automatically performed using Leica Biosystems for two sections of each block. For immunostaining, we used an automatic and clinically validated instrument (Ventana Benchmark Ultra Systems from Roche Tissue Diagnostics). For all antibodies, we used the automatic CC1 Ventana pretreatment (Cell Conditioning Solution, Ventana Catalog Number 950-124) for 52 min. We used two Ventana prediluted custom-fit antibodies, the anti-Neurofilament antibody (clone 2F11, mouse monoclonal, Roche Number 05267714001, by Cell Marque CMC26610021) and the anti-Glial Fibrillary Acidic Protein antibody (GFAP, clone EP672Y, rabbit monoclonal, Roche Number 05269784001, by Cell Marque CMC43450021). For NeuN, neuronal nuclear antigen, we chose the anti NeuN/Fox3 antibody (clone N/A60, mouse monoclonal, by Immunological Sciences MAB-90228; 1:100). All three antibodies were incubated for 1 h. The UltraView DAB procedure (Ventana Ultra System) was used for detection.

**Manual NeuN and Nestin immunostaining**

Manual immunohistochemistry was performed as previously described on the long-term fixation slides from the epileptic post-traumatic brain. Sections were dewaxed (2 washes in xylol for 10 min, 5 min in ethanol 100%, 5 min in ethanol 95%) and then rehydrated in distilled water for 5 minutes and in PBS 1x for 10 min. The neuronal nuclear antigen was unmasked using a commercially available kit (Unmasker, Diapath), according to the manufacturer’s instructions. We then employed the Dako CytoVision EnVision® + Dual Link System-HRP (DAB+) kit. After washing in PBS 1x, sections were incubated at room temperature for 10 min with Endogenous Enzyme Block, to quench endogenous peroxidase activity, and then incubated with the primary antibodies. For NeuN, we used the anti NeuN/Fox3 antibody (clone N/A60, mouse monoclonal, by Immunological Sciences MAB-90228; from 1:25 to 1:200). We used anti-nestin (mouse monoclonal, Immunological Sciences MAB-10430; 1:20), without unmasking, in the postnatal brain. For NeuN, we performed an overnight incubation at 4°C in humid atmosphere; for nestin, 100 minutes at room temperature in humid atmosphere. Slices were then rinsed twice in PBS 1x and incubated for another 30 min with Labeled Polymer-HRP (Dako CytoVision EnVision® + Dual Link System-HRP). The UltraView DAB procedure (Ventana Ultra System) was used for detection.

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**Figure 1.** Human dentate gyrus in post-mortem cases. Hematoxylin and eosin staining (HE), magnification 20x and automatized NeuN immunohistochemistry performed in four different fixation protocols obtained from the same autopic case, magnification 4x. A) HE in formalin fixation (classic protocol); B) HE in formalin fixation and formic acid treatment; C) HE in liquid fixative (LF) based on glacial acetic acid and formalin solution; D) HE in LF and formic acid treatment; E) NeuN staining in A protocol; F) NeuN staining in B protocol; G) NeuN staining in C protocol; H) NeuN staining in D protocol. HE staining shows that by B protocol the tissue matrix is more compact, without empty and irregular artefactual spaces, nuclei and nucleoli are more visible and better preserved and cell shape appears well represented (B). Automatized staining for NeuN results surely positive with little background exclusively in B protocol (F). Automatized NeuN immunostaining in fixation A protocol shows high level of background and paler staining for CA4 neurons (black arrow heads) and more loose parenchyma (E), compared to automatized NeuN staining in B protocol (F).
Link System-HRP (DAB+)]. The reaction product was detected as a brown substrate using 3,3-diamino-benzidinetetrahydrochloride (DAB). Finally, sections were mounted using a water-based mounting medium (Shur Mount™, TBS). The specificity of immunolabeling was verified with a control in which the primary antibody was omitted.

**Fluoro-Jade C (FJC) staining**

Sections were dewaxed as described above and incubated in a solution containing 1% NaOH in 80% ethanol (5 min), then in 70% ethanol (2 min), then in distilled water (2 min). They were then incubated for 10 minutes in 0.06% potassium permanganate, washed for 2 minutes in distilled water, and transferred to a 0.001% FJC staining solution (IS-0012, FJC solution 1000x, Immunological Sciences). After staining, sections were rinsed in distilled water, counterstained with 0.0001% 4',6-diamidino-2-phenylindole (DAPI) for 15 min, washed again, and air dried.

**Manual immunofluorescence**

Sections were dewaxed, rehydrated, and unmasked as described above. After washing in PBS, sections were incubated with Triton X-100 (0.3% in PBS 1x, room temperature, 10 min), washed twice in PBS 1x and incubated with 5% BSA and 5% serum of the species in which the secondary antibody was produced, for 30 min. They were then incubated with the primary antibodies overnight at 4°C, as follows: nestin (mouse monoclonal, Chemicon) and SOX2 (rabbit polyclonal, Immunological Sciences) 1:10 and 1:25 respectively. After 5-min rinses in PBS, sections were incubated with Triton X-100 (as above, 30 min), washed in PBS and incubated with the goat anti-mouse, Cy2 (Cyanine)-conjugated, secondary antibody (1:50 dilution; Jackson ImmunoResearch) for mouse primary antibodies, or with a goat anti-rabbit, Texas red-conjugated, secondary antibody (1:100; Jackson ImmunoResearch) for rabbit primary antibodies, at room temperature for 3.5 h. After staining, sections were washed in PBS, counterstained with 0.0001% DAPI for 15 min, and washed again. Coverslips were mounted using water-based anti-fading Gel/Mount (Biomeda).

**DNA/RNA extraction**

Total DNA/RNA were extracted using a total nucleic acid purification kit (RecoverAll Total Nucleic Acid Isolation Kit, Ambion Life Technologies, Foster City, CA, USA), from each paraffin embedded block by 10 µm thickness slices. The concentration and the quality of nucleic acid were investigated using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer. All of the procedures were produced according to our previously published protocols.30,31

**Analysis**

Sections were analysed using a Leica microscope (DMRA2), by three expert pathologists under double blind conditions. Cell shape, consistency of parenchymal...
matrix, nucleolar and nuclear preservation as well as immunohistochemistry positivity, have been evaluated using objectives from 5x to 20x by means a semiquantitative method based on score among 0 and +++. The degree of neurodegeneration was analyzed on Fluoro-Jade C sections, based on a modification of a previously described “neurodegeneration score” for animal models.32 The score range spanned from 0 (absence of Fluoro-Jade C-positive neurons) to +++ (maximal neurodegeneration pattern in the hippocampus). +++ was given with many positive cells with confluent fluorescence.

Results

We compared four different fixation protocols. Comparison was made between: A protocol (10% neutral-buffered formalin for 21 days); B protocols (10% neutral-buffered formalin for 21 days with two intervals of 1 hour each, at day 9 and 21, in which tissue was dipped in a 98% formic acid); C protocol (LF every four days and 70% ethanol for the last four days); D protocol (as C except at the day 9 and 17, when tissue was dipped in a 98% formic acid solution). We chose A protocol, traditionally utilized for most of the histochemical techniques, as our negative internal control to confirm and evaluate morphological observations obtained by others.

The automatically performed H&E staining was produced on the four differently fixed brain tissues, obtained from the same patient (Figure 1 A-D). We observed a better preservation of the cell shape, more compact parenchymal matrix and nucleolar and nuclear preservation with the formalin-formic fixation (B protocol, Figure 1B). For the perinatal and postnatal brain, characterized by a high content of water,33 we utilized only one approach of fixation, slightly modifying the fixation procedure of C protocol (C modified protocol). In fact, we used acetic acid addition to formalin with different ethanol steps, dehydrating agents. For immunostaining, we used an automatic and clinically validated procedure based on Ventana Benchmark Ultra systems from Roche Tissue Diagnostics. Both for NeuN and for easier to detect antibodies like GFAP, the quality of immunohistochemistry was superior by using the B protocol. Also the neurofilament and GFAP staining were better in B-type-tissues compared with the other fixations (Figures 1 E-H and 2 A,B).

Manual immunohistochemistry for NeuN (Figure 2 C-F) and nestin (Figure 4A) was performed on adult and postnatal cerebral tissues, respectively, by means a stronger and drastic manual unmasking protocol. We were able to perform an excellent staining on macroslices fixed in classic formalin protocol (A protocol), for adult brain tissue, whereas we utilized the previously described foetal-perinatal fixation protocol (C modified protocol), for the nestin immunostaining of postnatal brain tissue.

Regarding identification of the best antigen retrieval protocol, we verified different treatments: 1. CC1 Ventana pre-treatment; 2. microwave irradiation by means of a commercially available kit (Unmasker, Diapath); 3. formic acid pre-treatment; 4. Triton X-100 permeabilization; 5. a combination of 1 and 3, or 2 and 4.

We tested neurofilament (Figure 2C), GFAP (Figure 2D) and NeuN antigen expression in adult brains (Figures 1 E-H and 2 A,B,E-H) and Nestin and SOX2 in postnatal brain (Figures 4A and 5 A-E). For post-mortem adult brains, we applied automated and manual immunostaining. The first gave us the best results with formic acid and CC1 Ventana combination (1 and 3), suggesting that the B protocol is the preferable approach for automated antigen detection. Microwave irradiation and Unmasker Diapath solution produced an excellent result for manual NeuN detection in adult post-mortem tissue; however, the best fixation for this approach was the A protocol, exclusively formalin.

Concerning the postnatal brain, the unmasking procedure was necessary only for immunofluorescent staining, in which we utilized microwave irradiation plus Diapath Unmasker and Triton X-100 tissue permeabilization (2 and 4). Regarding Fluoro-Jade C staining, the A protocol for adults (Figure 3 A-F) and the C modified protocol for newborn tissues (Figure 4 B,C) has been utilized without...
any problems. Score for FJC positivity was totally in advantage for temporal lesion in post-traumatic epilepsy and for postnatal brain with hypoxic ischemic encephalopathy on the not-lesioned contralateral hippocampus (+++++) and respectively on SUID cases (+++/+). By means of this fixation method, concomitant with the immunofluorescence protocol, we detected neural stem cells positive for nestin and SOX2 (Figure 5 A-E).

Initial data of RNAs extraction and DNA amplification display apparently homogeneous and similar results with the four types of fixation (Table 3). Randomly we repeated the NeuN staining by automated Ventana procedure, on different post-mortem brain samples arrived to our attention for diagnostic purpose and obtained the same result.

### Discussion

The study of brain histology and protein expression represents a fundamental step in the understanding of brain development and physiopathology of the Central Nervous System diseases. Protein expression in FFPE tissue has been analysed, however, despite advances in molecular technologies, the quality of RNA and protein from fixed paraffin-embedded tissue remains variable. In particular, in autopic cases with long-term fixation and high variability deriving from the coroner post-mortem practices, both the immunohistochemistry and gene and RNA detection, request more accurate and detailed validation of custom fit procedures.7,8,34,35 Regarding to the preanalytical phase of post-mortem delay of autopsy, we choose the interval between death and autopic procedures around 40 hours. We standardized this interim period on 40 h, because it corresponds to the time of brain extraction that is considered adequate for the ethical problems (usually 24 h) and in which we do not observe any alteration in the intensity of staining of the more common neuronal and glial antigens (between 33 and 48 h).3 Recently, Thom and co-workers have published a review, in which they provide a framework for best practices, integration of clinical, pathological and molecular genetic investigations in Sudden Unexpected Death in Epilepsy (SUDEP).36 Authors underlined the lack of sufficient numbers of suitable control cases and tissues and lack of standardized guidance documents for conducting autopsies.37,38,39 Regarding to the preanalytical phase of post-mortem delay of autopsy, we choose the interval between death and autopic procedures around 40 hours. We standardized this interim period on 40 h, because it corresponds to the time of brain extraction that is considered adequate for the ethical problems (usually 24 h) and in which we do not observe any alteration in the intensity of staining of the more common neuronal and glial antigens (between 33 and 48 h).3 Recently, Thom and co-workers have published a review, in which they provide a framework for best practices, integration of clinical, pathological and molecular genetic investigations in Sudden Unexpected Death in Epilepsy (SUDEP).36 Authors underlined the lack of sufficient numbers of suitable control cases and tissues and lack of standardized guidance documents for conducting autopsies.37,38,39

![Figure 4. Human postnatal dentate gyrus in SIDS case. Nestin immunostaining, magnification 5x and Fluoro Jade C (FJC) neurodegeneration detection, magnification 20x. A) Nestin immunostaining in LF protocol: nestin-positive neural precursors are migrating from the subgranular (SGZ) and subventricular zone (SVZ); B) Human postnatal FJC staining (in green) of CA3 area of hippocampus in SIDS case, without any aspect of neurodegeneration, compared with CA3 area of perinatal FJC positive hypoxic ischemic encephalopathy in the control case (C), nuclei stained in blue by DAPI.](image)

### Table 3. RNA-DNA extraction.

| Fixations          | RNA, DNA |
|--------------------|----------|
|                     | 74/13    | 15/13 | D5/13 | 80/13 | 81/13 |
| A                   | ***      | ***   | **+  | /**   |       |
| B                   | **** *** | ***   |      |       |       |
| C                   | ***      | ***   |       |       |       |
| D                   | ***      | ***   |       |       |       |

RNA ratio: *1.55-1.64; **1.65-1.74; ***1.75-1.84; **** 1.85-1.99; DNA ratio: *1.55-1.64; **1.65-1.74; ***1.75-1.80.
from post-mortem brains in long-term fixation.

It is a consolidated and firm data that long-term formalin fixation for post-mortem human brain produces an impairment of immunolabeling with formalin-sensitive antibodies. Many expensive procedures have been yet tested without definitive good results. NeuN automatized immunostaining persists, for example, a prohibitive immunostaining for post-mortem brain tissues, as you can see by traditional protocol of formalin fixed brain macroslices (A protocol), in which NeuN staining carries on negative (Figure 1E). In other words, we chose A protocol as our negative internal control. Therefore, we decided to compare different substances to try to improve the staining results for formalin-sensitive antibodies. We considered formic acid and glacial acetic acid because respectively are normally utilized as antiprionic and dehydrating agents. Therefore, to determine the best strategy to maintain the integrity of post-mortem brain tissue, cell morphology and nuclear structure, we compared four different fixation protocols.

Comparison was made between: A protocol, exclusively formalin; B protocols, formalin and formic acid solution; C protocol, procedure with foetal autopsy dedicated LF, appropriate to dehydrate brain tissues and a mix method between B and C protocol (D protocol), to eventually test their synergism.

Preliminary data showed that the post-mortem brain tissue in formalin fixed and formic acid treated (B-type protocol) lone preserved good morphology in H&E. We scored histochemical results by microscope analysis of pathologists, however results were totally in favour of the B-type protocol. Tissue matrix is more compact, without empty and irregular artefactual spaces, nuclei and nucleoli are more visible and better preserved and cell shape appears well represented (Figure 1A-D).

Regarding identification of the best antigen retrieval protocol, it is well known that formalin and paraffin embedding both abolish or drastically reduce immunoreactivity with the majority of the antibodies used routinely in neuropathological studies, especially in autopic post-mortem tissues. We verified different treatments: 1. CC1 Ventana pre-treatment; 2. microwave irradiation by means of commercially available kit (Unmasker, Diapath); 3. Formic acid pre-treatment; 4. Triton X-100 permeabilization; 5. a combination of 1 and 3, or 2 and 4.

Our study evidenced that, for adult post-mortem brain tissue, the B protocol is the preferable approach for the automatized antigen detection, while A protocol, i.e. exclusively formalin, resulted the best fixation for manual immunostaining only when it is associated with a particular strong procedure of unmasking by microwave irradiation and Unmasker Diapath solution. Excellent automatized NeuN staining (Figures 1 E-H and 2 A,B) appears true positive with B protocol, totally negative by C and D fixation and false positive with classic A fixation. Indeed, granule cells of the dentate gyrus showed the same level of pale staining as astrocytes and glial cells (white arrow heads in Figure 2A), furthermore, neurons of CA4 area are NeuN positive.

Figure 5. Human postnatal neurogenesis in subventricular zone (SVZ) by antigen of stemness in SIDS case. Immunofluorescence staining in LF fixation protocol, magnification 20x. A) Nuclei in blue, DAPI; B) SOX2 in red; C) Nestin in green; D) merged image of A, B, C; E) Human postnatal neurogenesis in subventricular zone (SVZ) in SIDS case. Merged image of immunofluorescence staining; nuclei in blue, DAPI; nestin in green; SOX2 in red, in LF fixation protocol, magnification 40x.
exclusively by B fixation (black arrow heads in Figures 1 E,F and 2 A,B). In addition, GFAP and the neurofilament staining were better in B-type-tissues compared with the other fixations too (Figure 2 C,D). We consider our automated B protocol an interesting method to quantify clinical and research data from post-mortem tissue and a proficient approach to standardize NeuN detection according to shared validation system in different Institutions.\(^{36}\) Obviously, this approach has to be tested on long-fixed brains from brain bank collections. On the other hand, the manual protocol of immunostaining allows to control the development of the staining during the DAB exposure (checking under microscope) and reduce the problem of background (Figures 2 E-H). For the same reason the use of manual colorimetric staining could be suggested for paediatrics cases. Nevertheless, manual NeuN staining could be the first choice for dysplasia or brain cortical malformation and dysmorphism in perinatal deaths, an optimal approach to study the hippocampal sclerosis and granule cell pathology in post-mortem epileptic cases and SUDEP. The manual technique, more accurate than the automatic one, could be necessary to distinguish hypoplasia or dysgenesis of cardiorespiratory nuclei implicated in SIDS.

For the first time in the Literature, we demonstrated an optimal fixation with LF (C protocol) of postnatal brain. This type of protocol, in general, is utilized for embryonic and foetal brains because it has a great power of dehydration.\(^{37}\) In fact, according to Widdowson and Dickerson, the postnatal brain maintains a similar water content as foetus of 20-22 gestational weeks.\(^{38}\)

The striking decrease in brain water in adult aging resembles the decrease in brain water observed in early life through adulthood: 20-22 week foetus 92%, new-born 90% and adult 77%. By combined approach of double antigen retrieval in immunofluorescent staining, we distinguished nestin and SOX2 antigens of stemness in neurogenic area of postnatal brain, confirmed by colorimetric approach (Figure 4A).

There are very few data in the Literature regarding the development and application of novel histochemical tracer of neurodegeneration in human brain, for this reason we tested Fluoro-Jade C, usually utilized in animal models of neurodegeneration. According our result, we propose Fluoro-Jade C staining in adult and babies autopsies (Figure 3 A-F) as an easy and fast histofluostaining to suppose the presence of neurodegeneration cells in such amount as to exclude the sudden death.\(^{32,33}\)

Moreover, nucleic acid information does not seem lost through these four fixation techniques. Our current data of RNAs extraction show apparently homogeneous and similar results for the four types of fixation protocol and these outcomes are very interesting especially because it is possible to utilize LF and ethanol for perinatal brain tissue without lose the genomic information of cerebral samples. In fact, has to be considered mandatory for SIDS cases to realize the genomic analysis of possible channelopathies or potential eredopathies in infant sudden death. Furthermore, by our LF fixation protocol for postnatal brain tissue we were able to detect infective diseases utilizing Altona Diagnostics assay in efficient way (data not shown).

The processing parameters that determine the quality of RNAs and proteins in fixed paraffin-embedded tissues have been traditionally recognized. There are four important factors that are involved in RNA and protein preservation: time before fixation, methods of fixations, length of fixation and exclusively for protein detection, antigen retrieval techniques. The results of the different methods of fixation confirm that post-mortem brain tissue exhibited different affinity for antibodies or histo and/or fluorostaining and it is possible to adjust different protocols according to molecular requirement or to the patient ages. It is advisable in the future to test the different protocols for very long-lasting fixations, such as those of tissue banks, and drafting of the procedural guidelines.

References

1. Liu JY, Martinian L, Thom M, Sisodiya SM. Immunolabeling recovery in archival, post-mortem, human brain tissue using modified antigen retrieval and the catalyzed signal amplification system. J Neurosci Methods 2010;190:49-56.

2. Eisele S, Krumbholz M, Fischer MT, Mohan H, Junker A, Arzerberger T, et al. Prospects of transcript profiling for mRNAs and MicroRNAs using formalin-fixed and paraffin-embedded dissected autopic multiple sclerosis lesions. Brain Pathol 2012;22:607-18.

3. Carturan E, Testor DJ, Brost BC, Basso C, Thieme G, Ackerman MJ. Post mortem genetic testing for conventional autopsy–negative sudden unexplained death: An evaluation of different DNA extraction protocols and the feasibility of mutational analysis from archival paraffin-embedded heart tissue. Am J Clin Pathol 2008;129:391-7.

4. Wimmer I, Tröschler AR, Brunner F, Rubino SJ, Bien CG, Weiner HL, et al. Systematic Evaluation of RNA quality, microarray data reliability and pathway analysis in fresh, fresh frozen and formalin-fixed paraffin-embedded tissue samples. Sci Rep 2018;8:6351.

5. Marwitz S, Kolarova J, Reck M, Reinmuth N, Kugler C, Schädlich I, et al. The tissue is the issue: improved methylome analysis from paraffin-embedded tissues by application of the HOPE technique. Lab Invest 2014;94: 927-33.

6. Sas L, Van Laer S, Dierick AM, Duwel V, De Pauw A, Van Den Eynden G, et al. Study assessing the quality of quantification of estrogen receptor protein expression by immunohistochemistry and gene expression in breast cancer. Pathol Res Int 2014;2014:372653.

7. Engel KB, Moore HM. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. Arch Pathol Lab Med 2015;135: 537-43.

8. Lundström Y, Lundström P, Popova SN, Lindblom RPF, Alafouzoff I. Detection of changes in immunohistochemical stains caused by postmortem delay and fixation time. Appl Immunohistochem Mol Morphol 2018. doi: 10.1097/PAI.0000000000000658. [Epub ahead of print].

9. Thavarajah R, Mudimbamannar VK, Elizabeth J, Rao UK, Ranganathan K. Chemical and physical basics of routine formaldehyde fixation. J Oral Maxillofac Pathol 2012;16:400-5.

10. Battifora H, Kopinski M. The influence of protease digestion and duration of fixation on the immunostaining of keratins. J Histochem Cytochem 1986;34:1095-100.

11. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 1991;39:741-8.

12. Evers P, Uylings HBM. Effects of microwave pre-treatment on immunocytochemical staining of vibratome sections and tissue blocks of human cerebral cortex stored in formaldehyde fixative for long periods. J Neurosci Methods 1994;55:163-72.

13. Evers P, Uylings HBM. An optimal antigen retrieval method suitable for different antibodies on human brain tissue stored for several years in formaldehyde fixative. J Neurosci Methods 1997;72:197-07.

14. Hideaki K, Ryong-Woon S, Koichi O, Hiroyuki H, Shigeo M, Tetsuyuki K. Enhanced antigen retrieval of amyloid β immunohistochemistry: Re-evaluation...
of amyloid β pathology in Alzheimer disease and its mouse model. J Histochem Cytochem 2012;60: 61-9.
15. Brown P, Wolf A, Gajdusek DC. A simple and effective method for inactivating virus infectivity in formalin-fixed tissue samples from patients with Creutzfeldt-Jakob disease. Neurology 1990;40:877-90.
16. Liu JY, Martinian L, Thom M, Sisodiya SM. Immunolabeling recovery in archival, post-mortem, human brain tissue using modified antigen retrieval and the catalyzed signal amplification system. J Neurosci Methods 2010;190:49-56.
17. Pikkarainen M, Martikainen P, Alafuzoff I. The effect of prolonged fixation time on immunohistochemical staining of common neurodegenerative disease markers. J Neuropathol Exp Neurol 2010;69: 0-52.
18. Clarke JAL. Researches into the structure of the spinal cord. Phil Trans Roy Soc 1851;141:601-22.
19. Sririvasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002;161:1961-71.
20. Cimmino A, Parisi G, Mastropasqua MG, Ricco R. A new technique for foetal brain fixation and extraction. Pathologica 2002;94:320-24.
21. Righini A, Parazzini C, Doneda C, Avagliano L, Arrigoni F, Rustico M, et al. Early formative stage of human focal cortical gyration anomalies: fetal MRI. Am J Roentgenol 2012;198:439-47.
22. Schulz G, Crooijmans HJ, Germann M, Scheffler K, Müller-Gerbl M, Müller B. Three-dimensional strain fields in human brain resulting from formalin fixation. J Neurosci Methods 2011;202:17-27.
23. Shatil AS, Uddin MN, Matsuda KM, Figley CR. Quantitative ex vivo MRI changes due to progressive formalin fixation in whole human brain specimens: Longitudinal characterization of diffusion, relaxometry, and myelin water fraction measurements at 3T. Front Med (Lausanne) 2018;5:31.
24. Schmued LC, Stowers CC, Scallet AC, Xu L. Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. Brain Res 2005;1035:24-31.
25. Sarkar S, Raymick J, Schmued LC. The use of recently developed histochemical markers for localizing neurotoxicant induced regional brain pathologies. Toxins (Basel) 2014;26:1453-70.
26. Sarubbo S, Basso G, Chiofi F, Cesnik M, Paradiso B, Grandi E, et al. Technical, anatomical, and functional study after removal of a symptomatic cavernous angioma located in deep Wernicke’s territories with cortico-subcortical awake mapping. Case Rep Neurol Med 2013;2013: 835029.
27. Insauristi N, Amaral DG. Hippocampal formation. In: G. Paxinos, editor. The human nervous system. Elsevier, London; 2004.
28. Marconi P, Zucchini S, Berto E, Bozac A, Paradiso B, Bregola G, et al. Effects of defective herpes simplex vectors expressing neurotrophic factors on the proliferation and differentiation of neural cells in vivo. Gene Ther 2005;12:559-69.
29. Paradiso B, Marconi P, Zucchini S, Berto E, Binaschi A, Bozac A, et al. Localized delivery of fibroblast growth factor-2 and brain-derived neurotrophic factor reduces spontaneous seizures in an epilepsy model. P Natl Acad of Sci USA 2009;106:7191-6.
30. Roncon P, Zucchini S, Ferrari M, Marucci G, Giuliani M, Michelucci R, et al. Is autopsy tissue a valid control for epilepsy surgery tissue in microRNA studies? Epilepsia Open 2016;2:90-5.
31. Zucchi S, Marucci G, Paradiso B, Lanza G, Roncon P, Cifelli P, et al. Identification of miRNAs differentially expressed in human epilepsy with or without granule cell pathology. PLoS One 2014;9:e105521.
32. Zucchi S, Buzzi A, Bergamaschi M, Pietra C, Villetti G, Simonato M. Neuropeptidic activity of CHF3381, a putative N-methyl-D-aspartate receptor antagonist. NeuroReport 2002;13:2071-4.
33. von Bezold A. [Untersuchungen über die Vertheilung von Wasser, organischer Materia und anorganischen Verbindungen im Tiererreiche]. Z Wiss Zool 1857:8:487. [Article in German].
34. Matsuda Y, Fuji T, Suzuki T, Yamahatsu K, Kawahara K, Teda K, et al. Comparison of fixation methods for preservation of morphology, RNAs, and proteins from paraffin-embedded human cancer cell-implanted mouse models. J Histochem Cytochem 2011;59:68-75.
35. Zaglia T, Di Bona A, Chioato T, Basso C, Ausoni S, Mongillo M. Optimized protocol for immunostaining of experimental GFP-expressing and human hearts. Histochem Cell Biol 2016;146:407-19.
36. Thom M, Boldrini M, Bundock E, Sheppard MN, Devinsky O. The past, present and future challenges in epilepsy-related and sudden deaths and biobanking. Neuropathol Appl Neurobiol 2018;44:32-55.
37. Scagliotti V, Avagliano L, Gualtieri A, Grazio A, Doi P, Chalker J, et al. Histopathology and molecular characterisation of intrauterine-diagnosed congenital cranioopharyngioma. Pituitary 2016;19:50-6.
38. Widdowson EM, Dickerson JW. The effect of growth and function on the chemical composition of soft tissues. Biochem J 1960;77:30-43.