



**Susana Isabel  
Gonçalves Monteiro**

**Uso de Técnicas Imunohistoquímicas para  
Investigação de Espécimes de Museu de Doença  
Neurológica**

**Use of Immunohistochemical Techniques to  
Investigate Museum Specimens of Neurological  
Disease**



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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares, realizada sob a orientação científica do Professor Doutor Richard Greene do Departamento de Anatomia da University of Bristol e do Professor Doutor António Calado do Departamento de Biologia da Universidade de Aveiro.

To Christophe

*"Stones in the way? I keep them all...  
One day I am going to build a castle!"*

Fernando Pessoa (Portuguese poet and writer)  
in "A coragem de Pessoa"

## **o júri**

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## palavras-chave

Espécimes de Museu; Imunohistoquímica; Histologia; Neuropatologia; Preservação de longo-termo.

## resumo

Os espécimes de museu são um recurso valioso tanto para o ensino como para a investigação, mas geralmente têm sido utilizados para demonstrar a morfologia macroscópica. O objectivo da presente dissertação é determinar se modernas técnicas neuropatológicas, tal como a histologia e a imunohistoquímica, podem ser aplicadas com sucesso a espécimes de museu preservados por períodos superiores a 50 anos.

Poderá ser esperado que a qualidade do material histológico recuperado de espécimes de museu seja afectada por vários factores. Estes incluem a idade do espécime, o tipo e a duração da fixação/preservação, condições do acondicionamento do espécime, informação relativa ao paciente e o período *post-mortem*.

É importante compreender como é que os métodos de preservação mudaram com o passar dos tempos, conseqüentemente uma breve revisão desta história foi feita. É descrito o processo de selecção dos espécimes para este estudo e é apresentada uma investigação às características das diferentes soluções de montagem.

Grande parte desta dissertação refere-se ao desenvolvimento de formas que permitam a aplicação de técnicas histológicas modernas a espécimes de museu. É demonstrado que uma pós-fixação em formaldeído 10% e um ajuste do pH da solução corante de eosina para um intervalo entre 4.8 e 5.0, são especialmente importantes em tecidos envelhecidos.

Foi obtida uma coloração imunohistoquímica satisfatória utilizando o sistema EnVision+ (Dako), um polímero acoplado com HRP, com o anticorpo anti-neurofilamento (Dako). No entanto, apenas alcançou-se resultados satisfatórios com cortes de crióstato, os quais, aparentemente, produzem melhor coloração imunohistoquímica quando comparados com cortes de tecidos fixados em formaldeído e incluídos em parafina (FFPE), sugerindo assim, que o processamento em solventes orgânicos e as altas temperaturas da parafina derretida alteram a conformação do antígeno impedindo a detecção imunohistoquímica.

Um estudo detalhado de três casos datados de 1953, 1954 e 1955 confirma que técnicas modernas (incluindo imunohistoquímica) podem ser utilizadas em tecidos envelhecidos a um ponto de serem consideradas diagnosticamente válidas.

Este estudo demonstra que com ajustes cuidados aos protocolos é possível obter-se notáveis resultados histológicos de alta qualidade em tecidos que foram preservados por muitos anos. Confirma também, que espécimes de museu representam um valioso recurso para ensino e para a investigação a um nível ultraestrutural.

**keywords**

Museum Specimens; Immunohistochemistry, Histology, Neuropathology, Long-Term Storage

**abstract**

Museum Specimens are a valuable resource both for teaching and research but have generally been used to show the gross morphology. The aim of this dissertation is to determine if modern neuropathological techniques, such as histology and immunohistochemistry, can be applied successfully in museum specimens that have been preserved for periods in excess of 50 years. It may be expected that many factors could affect the quality of histological material retrieved from museum specimens. These include the age of the specimen, type and length of fixation/preservation, storage conditions, patient information and *post-mortem* period.

It is important to understand how preservation methods had changed over time, and therefore, the complex history of "potting" is reviewed. The process of selecting specimens for this study is described and an investigation into the characteristics of mounting solutions is presented.

The major part of the dissertation concerns the development of ways in which to apply modern histological techniques to museum specimens. It is shown that post-fixation in formaldehyde 10% and an adjustment of the eosin stain solution to a pH ranging between 4.8 and 5.0 are especially important in older tissues.

Satisfactory immunohistochemistry staining was obtained using EnVision + (Dako) system, a HRP labeled polymer, with anti-neurofilament antibody (Dako). Nevertheless, successful results were only achieved with cryostat sections, as they appeared to produce better immunohistochemistry staining when compared to formalin-fixed paraffin-embedded (FFPE) sections, suggesting that processing in organic solvents and high temperatures of molten paraffin alters the conformation of the antigen hampering immunohistochemistry detection.

A detailed study of three cases from 1953, 1954 and 1955 confirms that modern techniques (including immunohistochemistry) can be used in aged tissue to the point where they are useful diagnostically.

This study shows that with careful adjustment to protocols it is possible to achieve remarkably high quality histological results in tissues that have been preserved for many years. It confirms that specimens in museums represent a valuable resource for teaching and research at an ultrastructural level.

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## **1. PATHOLOGY MUSEUM SPECIMENS**

## **1. PATHOLOGY MUSEUM SPECIMENS:**

### **1.1 Contribution to anatomical and pathological study**

Museum mounted specimens are a valuable teaching resource. They contribute to a better understanding of human anatomy and pathology. Studying anatomical and pathological specimens in conjunction with histological and other types of molecular information will provide students with the opportunity to understand anatomy and/or pathology in a clinical context, using clinical reasoning. Attempting different techniques, such as histology and immunohistochemistry, in museum specimens may therefore allow correlation between the microscopic and macroscopic features in both anatomical and pathological conditions.

Furthermore, museum specimens may also be a useful tool for research. The availability of human tissue for research has been decreasing and the number of human bodies donated is insufficient for research needs ([www.hta.gov.uk](http://www.hta.gov.uk)). This decrease may be in response to the imposition of the Human Tissue Act of 2004 that repeals and replaces the Human Tissue Act 1961, the Anatomy Act 1984 and the Human Organ Transplants Act 1989. The Human Tissue Act of 2004 is even stricter on the control of body donation as it requires consent to be both written by the donor and witnessed ([www.hta.gov.uk](http://www.hta.gov.uk)). In addition, the number of medical schools in Wales and England has increased, therefore the combination of an increased number of students at individual medical schools and reduced body donation also contributes for this shortage of cadavers for education and training of medical students and researchers ([www.hta.gov.uk](http://www.hta.gov.uk)).

A retrospective analysis of archival post-mortem specimens is a very useful tool for studying pathological conditions, and studies of human post-mortem specimens may lead to the generation of new hypotheses on the aetiology and pathophysiology of diseases. During the last 20 years many groups of investigators have attempted different molecular techniques with archival post-mortem tissue. Archival tissue refers to old-preserved tissue and it could be preserved in liquid fixative (e.g. tissue banks), paraffin blocks, sections attached to a glass slide, or museum specimens preserved in preservative fluid.

In 1988, Wieczorek and colleagues used 105 archival pathological specimens of lymphoid neoplasia for demonstrating neoplastic T cells using monoclonal antibody Leu-22. This led the way for numerous publications referring to the use of immunohistochemistry techniques using archival specimens. Also in 1988, Shibatta and his colleagues performed an analysis of DNA sequences in forty-year-old paraffin-embedded thin-tissue sections with success. Extraction of DNA from formalin-fixed, paraffin embedded tissue for PCR analysis has also been well documented (Jackson *et al.* 1990; Forsthoefel *et al.* 1992; Frank *et al.* 1996).

RNA was first extracted from formalin-fixed, paraffin-embedded tissue for Northern and dot-blotting analysis in 1988 (Rupp and Locker 1988). Subsequently, many reports were made about extraction of viral or human cellular RNA from archival samples and successful amplification of extracted RNA has also been reported (von Weizacker *et al.* 1991; Finke *et al.* 1993; Koopmans *et al.* 1993; Goldsworthy *et al.* 1999; Masuda *et al.* 1999; Korbler *et al.* 2003; Byers *et al.* 2004). A number of studies have also indicated that RNA extraction from post-mortem tissue can be hampered due to delay before fixation, prolonged fixation, or long-term preservation after fixation (Bresters *et al.* 1994; Cronin *et al.* 2004). It is believed that the low efficiency of RNA extraction is secondary to cross-linking of proteins (Finke *et al.* 1993; Park *et al.* 1996), and impaired reverse transcriptase reaction (Masuda *et al.* 1999) by formalin induced modification (addition of mono-methylol to amino groups of four bases) of extracted RNA (Feldman, 1973; Auerbach *et al.* 1977; Masuda *et al.* 1999, Hamatani *et al.*, 2006).

More recently, the study of proteins using mass spectrometry has also been attempted with archival post-mortem specimens. Fetsch and colleagues (2002) used Surface-Enhanced Laser Desorption/Ionization (SELDI) affinity mass spectrometry in cytological archival preparations; Bhattacharya and colleagues (2003) used laser capture microdissection Matrix-Assisted Laser Desorption/Ionization (MALD); in 2005, Crockett and colleagues identified proteins from formalin-fixed paraffin-embedded cells by Liquid-Chromatography/Mass Spectrometry/Mass Spectrometry (LC-MS/MS); and in 2006, Shi and colleagues

assessed the quality of protein extraction from formalin-fixed, paraffin-embedded tissue sections by mass spectrometry.

Archival specimens have also been used in neuroscience research. Adams and Poston (1990) analysed the chronicity of lesions in brains affected by multiple sclerosis using both archival and recent post-mortem tissue. The antigen expression in brain specimens and tumours has been studied by several investigators (Banerjee *et al.*, 1997, Horuk *et al.*, 1997, Ylagan *et al.*, 1997, Laeng *et al.*, 1998, Sheaffer *et al.*, 1999, McLendon *et al.*, 2000, Ferrer *et al.*, 2000, Van Deerlin *et al.*, 2002) and, in 1998, Dwork and his colleagues studied the use of archival, formalin-fixed tissue in their analysis of changes in Alzheimer's type. These publications demonstrate the feasibility of using archival post-mortem human tissue in research for molecular studies. However, the use of museum specimens has not been investigated apart from DNA studies for phylogenetic studies of species and conservation genetics research, using animal or plant collections (Austin and Melville, 2006, Barnes *et al.*, 2000).

## **1.2 Variables affecting specimen condition:**

Tissue from museum specimens presents disadvantages when compared to more recently collected tissue. These are derived mostly from the effects of fixation/preservation, artefacts due to ageing, and storage conditions. Therefore conventional histological or molecular methods in such tissue may be hampered due to possible degradation of the tissue under such conditions.

Most of the pitfalls of studying museum specimens are related to a lack of information about the specimen and to a failure to control for the effects of long-term storage and preservation. To minimise the possible negative contribution of these variables, it is essential to gather as much information as possible about each specimen. For example, knowing the age of specimen is essential, especially in retrospective studies, but this is not always stated on the specimen or pot.

Technical information about storage conditions should be also given. If the potted specimen was subjected to direct light, this could cause artefacts leading to

a decrease in levels of preservation and changes in the specimen's colour. If the specimen has been re-potted several times, it is possible that it has been submerged in different mounting fluids, thereby changing preservation conditions. Another factor to bear in mind is the fact that every time a pot is opened, the mounting fluid becomes more acidic due to oxidation by ambient air that lowers pH levels (Barnes *et al.* 2000).

It is well established that fixation is the only stage which is irreversible, and that, if it is not well performed, it could compromise all further steps. Therefore, the type of fixative used, whether other fixatives have been used, the method, the length of fixation time, and all other conditions related to fixation should be extensively known. One of the drawbacks of using archival specimens is that it is not possible to control these fixation variables. However, understanding all fixation conditions will help to track pitfalls.

Knowing the origin of the pot and the name of the technician who made it is always a good solution since most of the time he/she possesses all the information previously mentioned.

The delay between death and subsequent post mortem (P.M.) examination and harvest of specimens is another factor which needs to be controlled. Delays in tissue fixation often lead to artefacts which could be confused with pathological features. A detailed patient history is also important: age, sex and pre-clinical conditions are some examples of variables which need to be controlled for successful studies.

### **1.3 Aims of the present study:**

There are many thousands of anatomical and pathological specimens in museums and storage. The main aim of the present study is to know if material from museum specimens can be processed using modern neuropathological techniques to a state where it would be of diagnostic value or use in teaching and research.

This study will test the following hypotheses:

(i) Tissue from museum specimens can be used in histological studies with useful results.

Although previous studies clearly show that prolonged fixation will cause molecules to undergo conformational changes, hampering molecular detection, with museum specimens this issue could be reduced as most of the specimens were fixed first and then stored in preservative fluid.

(ii) Tissue from museum specimens can be used in immunohistochemical studies with useful results.

Prolonged fixation/preservation could destroy the antigenicity of the tissue. Although it is well known that formaldehyde fixation reduces immunoreactivity, a number of epitope retrieval techniques have been developed, thus increasing the antigen detection.

## **2. BRIEF HISTORY OF METHODS OF ORGAN PRESERVATION**

## 2. BRIEF HISTORY OF METHODS OF ORGAN PRESERVATION:

The first evidence of the deliberate preservation of human organs comes from Ancient Egypt and dates from 2750 B.C. During mummification, organs were removed (apart from the heart) and placed in canopic jars to dry in a mixture of salts (mostly sodium carbonate and sodium bicarbonate) called *natrom*. Organs were preserved because Egyptians believed that the body was linked with the afterlife (<http://www.ancient-egypt.org/index.html>) (Fig 2.1).

Over time, preservation methods and potting techniques have developed, and organs were preserved for medical studies. In the early 18<sup>th</sup> Century names of pathologists such as Fredrik Ruysch (1638-1731) and William Hunter were highlighted by their contribution to organ and tissue preservation with their extensive collections. The first collections of museum specimens were “spirit collections” where the specimens were preserved in an alcoholic fluid. The first alcoholic preservative fluid was a crude distillate of spirit of wine of around 40% concentration (Down, 1989). The containers were made of glass, of a cylindrical shape and sealed by the use of bitumen and gutta percha-soaked pig bladder (Barnes *et al.*, 2000). Some years later, the cylindrical containers were replaced by glass containers of rectangular shape. This type of format eliminates visual distortion caused by cylindrical shape (Fig 2.1).

In the 1950s *Perspex*, a cast acrylic sheet, with the same refractive index as glass but much lighter and less fragile was introduced as a material to make pots. Its properties make the handling of the pot easier. The first publication on how to make pots with *Perspex* was made in 1958 by Proger (Proger, 1958) (Fig 2.1). However, not only had the type of pots developed, but also the preservative fluids.

### 2.1 Fixative/Preservatives fluids:

Museum specimens can be preserved in many different fluids, and some of these fluids may vary according to the curator. Moreover, some curators have their

## 2. BRIEF HISTORY OF METHODS OF ORGAN PRESERVATION:

own “recipes” without publishing these at all. They are described in Fig 2.1 as “secret solutions”. Museum fixative/preservative fluids can be grouped into three main categories: (a) aldehydes, such as formaldehyde or glutaraldehyde, (b) alcohols, in different concentrations and sometimes mixed with other components, and (c) Kaiserling solution (Kaiserling, 1897). Some act as a fixative and others as a preservative.

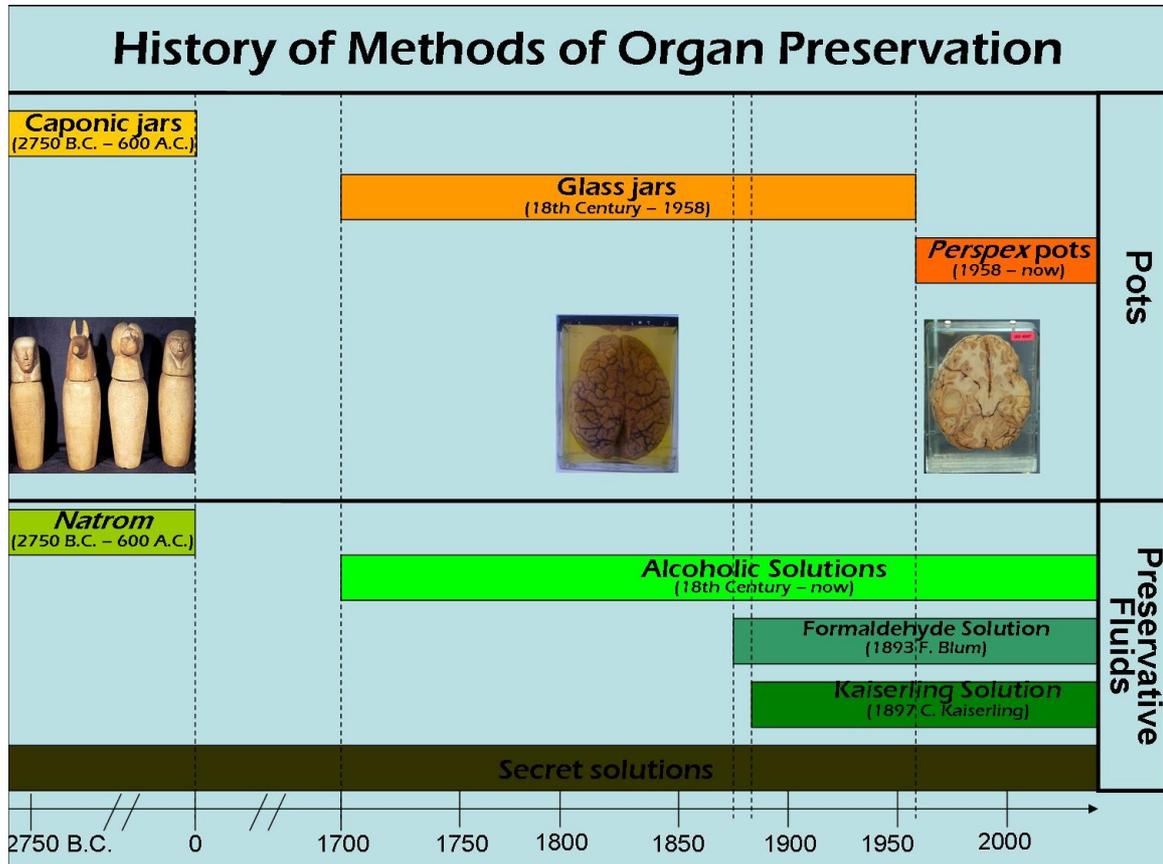
A fixative will allow tissue to retain, to a large extent, its appearance in life by stabilizing proteins by cross-linking, and also by inactivating enzymes and therefore stopping autolysis. However, specimens should not be stored in an aldehyde fixative, like formaldehyde, for a long time since they become brittle and molecules suffer dramatic conformational changes. A preservative is a chemical in which the specimen can be stored without further degradation. The main role of a preservative is to provide an environment that eliminates bacterial and fungal growth.

The first “wet specimens” preparations were made in the early 18<sup>th</sup> Century. Specimens were preserved in alcoholic-based fluids (Fig 2.1). Alcohol is a preservative but does not fix the tissue so the interior of the specimen could slowly start to degrade in large specimens. Alcohol also causes a discoloration on specimens colour and shrinkage.

In 1893 (Fig 2.1), Ferdinand Blum, a young German doctor, discovered not only that formaldehyde is an effective agent for killing bacteria (Blum, 1893a), but also that it hardens tissues in the same way that alcohol does, and that when these tissues were prepared for histology, the staining quality was superior (Blum, 1983b). Moreover, formaldehyde produced minimum shrinkage and distortion of the tissue when compared to alcohol-preserved tissues (Blum, 1893a, Blum, 1983b).

Specimens preserved in alcohol or aldehyde-based solutions for prolonged periods of time tend to lose their natural colour, so in 1897 Carl Kaiserling published a novel method of preservation for museum specimens (fig 2.1) that also preserves their colour (Kaiserling, 1897). This new revolutionary method has been used with different variations, but the basic principle consists of prior fixation of the specimen with formaldehyde before the specimen is placed in a solution

with potassium acetate (preservative), glycerol (for the softening of the tissue and to increase refractive index), and an anti-microbial agent such as thymol (Kaiserling, 1897).



**Fig 2.1 – History of Methods of Organ Preservation.** 2750 B.C. - Organs were dry in *natrom* and preserved in caponic jars. 18<sup>th</sup> Century – Specimens were preserved in alcoholic solutions in glass jars. 1893 – Ferdinand Blum discovered the fixatives properties of Formaldehyde. 1897 – Carl Kaiserling introduces a new preservative method with Kaiserling solution. 1958 – First publication on how to make pots with Perspex (Proger, 1958).

As it is possible to see in fig 2.1, the history of methods of organ preservation is complex and some of the events are not well documented. As it is shown in fig 2.1, the mounting fluids now in use may vary, and the use of “secret solutions” by some curators complicates even more the process of identifying the preservative fluid.

### **3. IDENTIFICATION OF THE SPECIMENS**

### **3. IDENTIFICATION OF THE SPECIMENS**

#### **3.1 Introduction:**

The Institute of Anatomical Sciences and the Department of Anatomy at the University of Bristol, are responsible for a pot reclamation scheme that involves the collection and storage of specimens from institutions looking to either downsize or dispose of their collections. Before histological processing and immunohistochemistry it was necessary to organize all the information about each specimen and select some samples for further study.

#### **3.2 Material and Methods:**

At first, all information available about each specimen was gathered in tabular form. A macroscopic description was produced detailing the lesions in 219 specimens from the nervous system. Specimens were excluded from the study if they lacked information about the specimen age.

After analysing the appearance of each specimen taking into account the colour of the specimen and the mounting fluid, any signs of putrefaction or dryness (if there was any part of the specimen not immersed in mounting fluid), 17 specimens were randomly selected. The oldest was originally potted in 1953 and the most recent one in 2007 (Table1). The 2007 specimen was used as a positive control for the histological and immunohistochemical studies, because it was least likely to have suffered any degradation of tissue integrity due to prolonged fixation/preservation.

### 3. IDENTIFICATION OF THE SPECIMENS

**Table 3.1**

ID	Year	Mount. Fluid (M.F.)	pH M.F.	Type of Pot	Provenance
5883	1953	Kaiserling	N/A	<i>Perspex</i> Pot	Worcester
5339	1954	Kaiserling	N/A	<i>Perspex</i> Pot	University of Birmingham
5202	1955	Kaiserling	8.13	<i>Perspex</i> Pot	University of Birmingham
E 143	1957	Aldehyde (+100mg/L)	7.24	<i>Perspex</i> Pot	Edinburgh University
E 142	1959	Alcoholic	6.95	<i>Perspex</i> Pot	Edinburgh University
E 144	1960	Aldehyde (+100mg/L)	6.81	<i>Perspex</i> Pot	Edinburgh University
5497	1964	Kaiserling	5.89	<i>Perspex</i> Pot	University of Birmingham
E 104	1965	Aldehyde (40mg/L)	7.11	<i>Perspex</i> Pot	Edinburgh University
5423	1968	No sample	N/A	<i>Perspex</i> Pot	University of Birmingham
345	1968	Alcoholic	7.88	<i>Perspex</i> Pot	UCL Pathology Museum
4424	1969	Aldehyde (60mg/L)	7.58	<i>Perspex</i> Pot	Worcester
5425	1970	Aldehyde (+100mg/L)	7.48	<i>Perspex</i> Pot	University of Birmingham
4406	1980	Aldehyde (60mg/L)	6.76	<i>Perspex</i> Pot	??
1987	1987	2% Formalin	4.50	Plastic Bucket	University of Bristol
1995	1995	2% Formalin	4.36	Plastic Bucket	University of Bristol
2002	2002	2% Formalin	4.19	Plastic Bucket	University of Bristol
2007	2007	2% Formalin	N/A	Plastic Bucket	University of Bristol

**Tab 3.1 – Specimens Included in the Study.** Seventeen specimens were selected, dated from 1953 to 2007. There was at least one sample from each decade. Each of the mounting fluids was identified and the pH measured

**Table 3.2**

	<b>Year</b>	<b>Macroscopic diagnosis</b>
5883	1953	Cystic Astrocytoma
5339	1954	Craniopharyngioma? Giant Aneurysm?
5202	1955	Brain Stem Glioma
E 143	1957	Abscess
E 142	1959	Astrocytoma? Glioblastoma?
E 144	1960	Malignant Astrocytoma of Pineal
5497	1964	Astrocytoma
E 104	1965	??
5423	1968	Encephalitis
345	1968	Glioma?
4424	1969	Paraventricular Glioma
5425	1970	Astrocytoma?
4406	1980	Glioma
1987	1987	Cerebrovascular accident
1995	1995	Brain Stem Stroke
2002	2002	Bronchopneumonia
2007	2007	Non-Neurological

**Tab 3.2 – Specimens Included in the Study.** Many of the pathological specimens available in the museum archive were supplied with an indication of possible underlying diagnosis. A number of cases spanning the years 1953-2007 were selected.

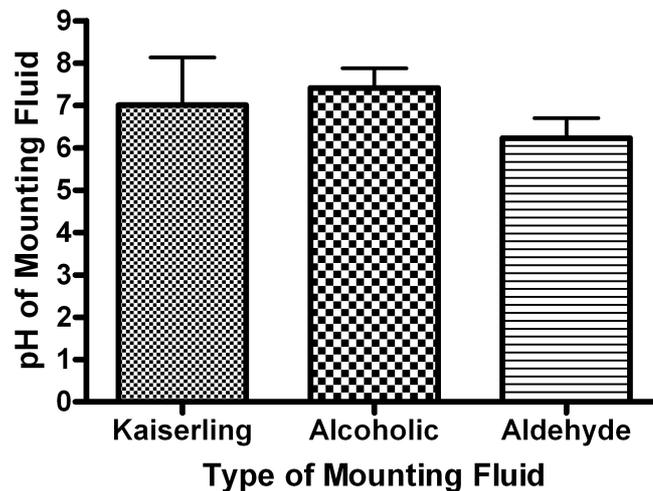
Thirteen specimens were selected for mounting fluid study. One sample of the mounting fluid in each pot was saved in order to study its characteristics. The type of fixative/preservative fluid, in the cases 5883, 5339, 5202, 5497 was confirmed by telephone and/or email contact with the curators and museum technician of each institution. The presence of formaldehyde was detected by Test Strips (MerckoQuant®). The pH was measured with Whatman® PHA 230 pH meter. Values of pH were statistically treated for the analysis of the mean pH using One-way Analysis of Variance (ANOVA) and also, to see if there is any significant correlation between pH and the number of years those specimens were immersed in the mounting fluids, using a linear regression analysis. All the statistic work was done using GraphPad Prism® Version 4.03.

**3.3 Results:**

Identification of mounting fluids has revealed that two specimens were preserved in Kaiserling solution, two in an alcoholic solution and nine in an aldehyde solution. The mean pH for Kaiserling solutions was of 7.010, for the alcoholic solutions was of 7.415 and for the aldehyde solutions was of 6.226.

One-way Analysis of Variance (ANOVA) revealed no statistically significant difference in the mean pH of the different mounting fluids examined ( $p=0.5024$ ) (Fig 3.1).

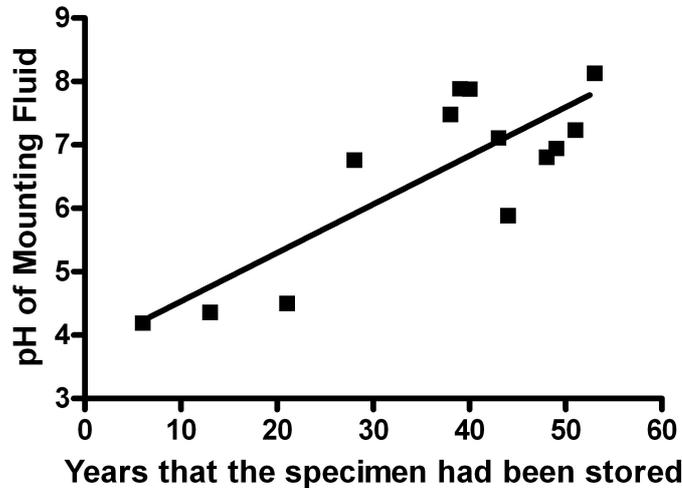
**pH of Mounting Fluid versus Type of Mounting Fluid**



**Fig 3.1 – Mean pH of Mounting Fluids Versus Types of Mounting Fluids.** Error bar = standard error of the mean (SEM). Kaiserling solution  $n = 2$ , alcoholic solutions  $n = 2$  and aldehyde solution  $n = 9$ . ANOVA,  $p = 0.5024$ .

The pH of the thirteen solutions of different age was compared and linear regression analysis has shown that as the number of years that the specimen is stored increases the solution is more alkaline (Fig 3.2).

### Age of the specimen versus pH of Mounting Fluid



**Fig 3.2 – Relationship Between Age of Specimen and pH of Mounting Medium.** N = 13. Linear regression analysis  $p \leq 0.0005$ .

#### 3.4 Discussion/Conclusions:

The museum archive includes 219 pathological specimens showing macroscopic evidence of brain pathology. From these cases, it was possible to establish the age of the specimen in only 50 cases. From this group, investigation of mounting fluid properties was investigated in 17 randomly selected cases.

The type of mounting fluid varies according to the age of the specimen. This may reflect the preferences of different museum curators regarding the choice of mounting fluid. The preservative detected in the majority of the specimens was aldehyde based, although it is known that Kaiserling solution is the mounting fluid mostly used nowadays. This discrepancy can be explained by the fact that some of the original mounting fluids were being substituted by formaldehyde solution for a post-fixation, prior to preservation in Kaiserling solution. Therefore, some of the mounting fluids analysed are probably not the originals.

There was no statistically significant ( $p \leq 0.05$ ) difference between the mean pH of the three solutions ( $p = 0.5024$ ). It should be noted that pots in solutions other than aldehydes, are comparatively difficult to obtain hence the small “n” values.

### 3. IDENTIFICATION OF THE SPECIMENS

The search for further examples is being continued ahead of making any definitive conclusions.

However, there is a significant positive correlation between pH and age of the specimens ( $n=13$ ,  $p=0.0005$ ). It appears that the pH of the mounting fluid increases with increasing age of the specimen. It is of note that the four most recent samples were all preserved in 2% formaldehyde, and that these have the lowest pH values. It is also interesting to note that although the use Perspex pots was first reported in 1958 (Proger, 1958), the specimens that dated from 1953, 1954, 1955 and 1957 were potted in Perspex pots (Table 3.1). This suggests that these specimens may have been re-potted and possibly preserved in different conditions over the course of their history.

These results show the need of increasing the number of specimens analysed for the significance of the values and further studies on the characteristics of mounting fluids.

#### **4. HISTOLOGICAL METHODS USING MUSEUM SPECIMENS OF THE C ENTRAL NERVOUS SYSTEM**

## **4. HISTOLOGICAL METHODS USING MUSEUM SPECIMENS OF THE CENTRAL NERVOUS SYSTEM:**

### **4.1 Introduction:**

Conventional histological methods using museum specimens may be hampered because of factors such as specimen age and state of preservation (see General Introduction).

The primary aim of this first set of experiments is to develop optimal tissue processing and staining methods for use in museum specimens of brain tissue.

### **4.2 Material and Methods:**

A brain specimen that has been preserved in Kaiserling solution for approximately 55 years was used. Six blocks of tissue (measuring approximately 10x10x10mm) were sampled from the right frontal lobe and included cortex and superficial white matter. The samples underwent different pre-treatments and processing cycles and were sectioned using different thickness.

#### 4.2.1 Pre-Treatment:

Following removal from the museum preservative fluid, two of the tissue samples were washed and subjected to overnight post-fixation in 10% formaldehyde diluted in water; another two samples were washed in running tap water overnight, and a further two samples were not pre-treated, going directly to the first processing alcohol (70% methanol).

#### 4.2.2 Processing cycles:

Three samples (each one exposed to one of three different pre-treatment – see above) underwent two different processing cycles.

(i) A processing cycle of twelve hour was tested. The tissue samples were immersed of the samples in graded alcohols for the dehydration (70% methanol for an hour, 90% methanol for 1 hour, methanol for 1 hour and absolute ethanol for 1 hour). The next stage was the clearing and preparation of the samples for paraffin impregnation. The samples were placed in three solutions of low toxicity xylene substitute (Metaclean Solvo 8000 from Merck) for 1 hour each. Finally, the samples were impregnated with molten liquid paraffin (+60°C) in three different solutions (1 hour each and 2 hours on the last paraffin). All the stages of the twelve hour processing cycle were performed manually under agitation using a rocket shaker (Platform Rocker STR 6 from Stuart Bibby).

(ii) A processing cycle of twenty-four hours was tested. The samples were dehydrated in grading alcohols (methanol 70% for 2 hours, alcohol 90% for 2 hours, methanol for 2 hours and absolute ethanol for 2 hours). They were cleared with a low toxicity xylene substitute (Metaclean Solvo 8000 from Merck) three times for 2 hours each, and finally impregnated with molten paraffin (+60°C) for 3x2 hours. A processing machine was used (2L Processor MkII from Shandon Southern) and vacuum was used in the last impregnation with paraffin in order to increase the paraffin penetration in the tissue.

#### 4.2.3 Microtomy:

The samples were embedded in paraffin blocks, and sections were cut at 3µm and 5µm using a microtome (Reichert-Jung 2050 from Leica). All the sections were stained using the H&E staining method. They were regressively stained with Erlich haematoxylin for three minutes and eosin (adjusting pH to 4.8-5.0) for 2 minutes. After dehydration (methanol 70% for 3 minutes, methanol for 3 minutes and ethanol for 3 minutes) and clearing (xylene Fisher Scientifics 2 X 3minutes), they were mounted using a resin medium (DPX from Lamb).

#### 4.2.4 Histological assessment:

Photomicrographs of the slides were made and compared with photomicrographs of a recent sample from 2007 used as a control, from the same

region of the brain. All the slides were blindly assessed for the integrity of the tissue and quality of staining. The results were compiled in a table.

### 4.3 Results:

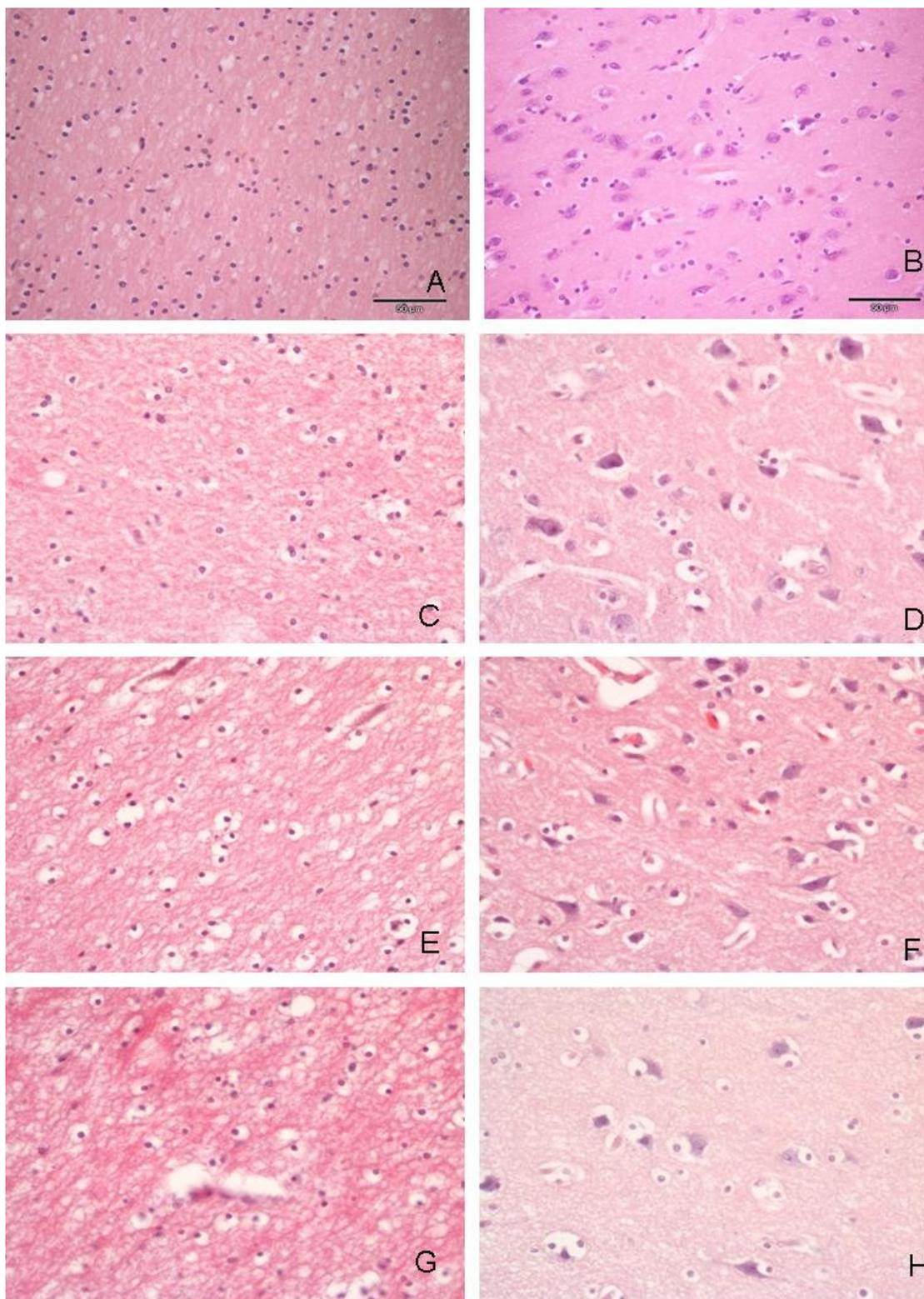
Samples that underwent post-fixation in 10% formalin produced sections that revealed an adequate processing, no evidence of shattering, and were evenly stained (Tab 4.1) The samples that were not subjected to any pre-treatment revealed an uneven stain and an inadequate processing (Tab 4.1). Samples that were washed in running water overnight produced sections with evidence of shattering and cracking, and in some cases the staining was uneven (Tab 4.1). The majority of the sections 5µm thick were stained more intensely by eosin (Tab 4.1).

Sections	Processing	Microtomy		Stain-general		Haematoxylin		Eosin
	Adequate/Inadequate	Shatter	cracking	uneven	stain deposit	intensity	chromatin	Intensity
N/12/3µm	Inadequate	No	No	Yes	No	+++	+++	++
N/12/5µm	Inadequate	No	No	Yes	Yes	+++	+++	++
N/24/3µm	Adequate	Yes	Yes	Yes	No	+++	+++	++
N/24/5µm	Adequate	Yes	Yes	Yes	No	+++	+++	+++
F/12/3µm	Adequate	No	Yes	No	No	+++	+++	+
F/12/5µm	Adequate	No	Yes	No	No	+++	+++	++
F/24/3µm	Adequate	No	Yes	No	No	+++	+++	+++
F/24/5µm	Adequate	No	Yes	No	No	+++	+++	++
W/12/3µm	Adequate	Yes	No	No	No	+++	+++	++
W/12/5µm	Adequate	Yes	Yes	No	No	+++	+++	+++
W/24/3µm	Adequate	Yes	Yes	Yes	No	+++	+++	++
W/24/5µm	Adequate	No	Yes	Yes	No	+++	+++	++

**Table 4.1 – Assessment of Histological Sections.** All stained slides were assessed blind for the adequacy of the processing, artefacts due to microtomy, general features of the stain and for the features of the haematoxylin and eosin staining.

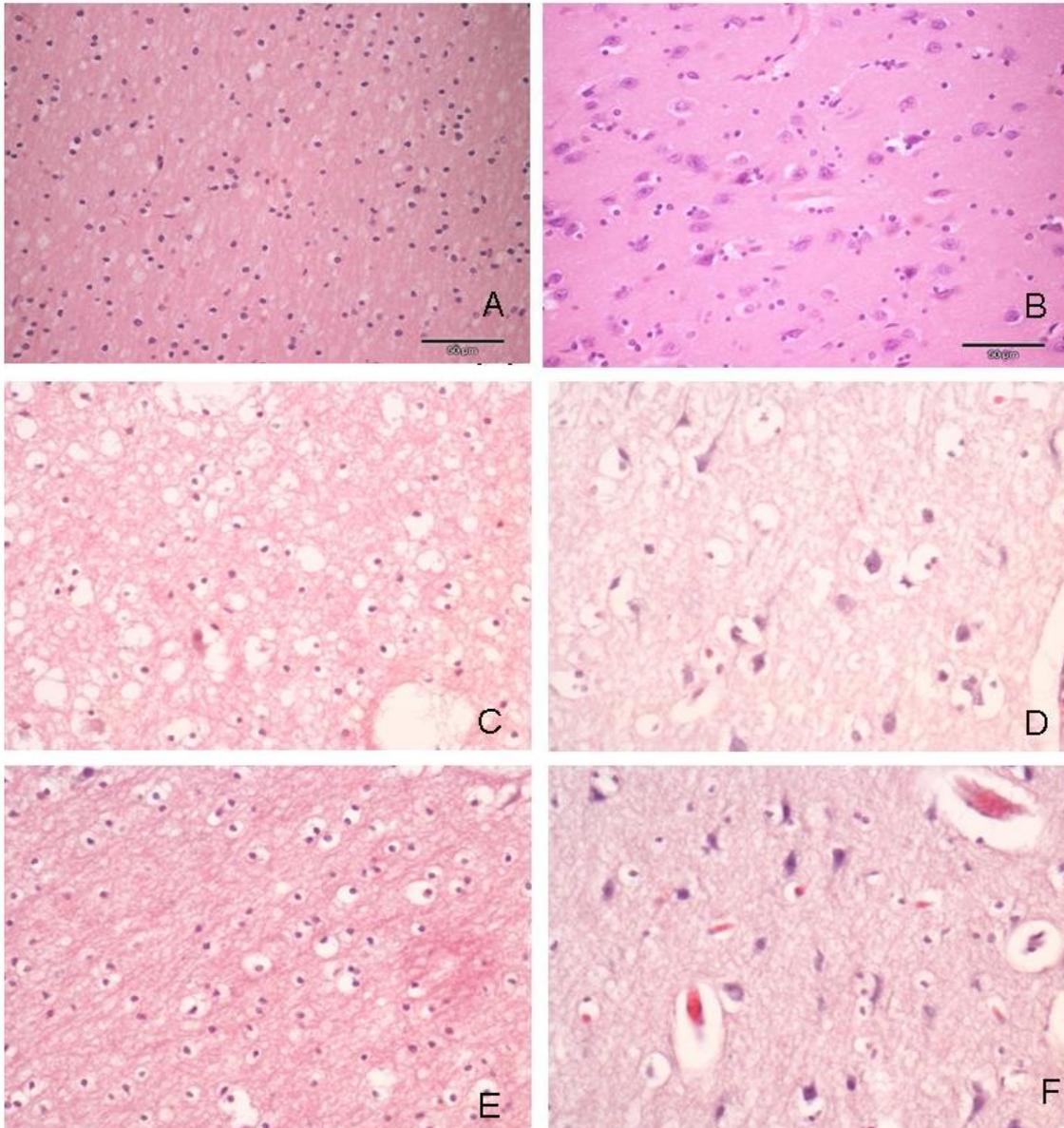
We have used a specimen from 2007 showing white matter (Fig 4.1 A) and cortex (Fig 4.1 B) as a standard against which to compare the older specimens. Samples post-fixed in 10% formalin had produced similar results (4.1 C and D)

comparing to the standard sample. However, in Fig 4.1 D is possible to see that nuclear detail, as chromatin and nucleoli, is not as distinctive as in the standard sample (Fig 4.1 B). The samples that have not been pre-treated had shown less preservation of the tissue (Fig 4.1 E and F). Loss of preservation was even worst in samples that were washed in running tap water overnight (Fig 4.1 G and H). 5 $\mu$ m thick sections were stained more intensely (Fig 4.2 E and F) compared to 3 $\mu$ m thick sections (Fig 4.2 C and D). Photomicrographs of the standard sample of 2007 are shown for the comparison of histological qualities.



**Fig. 4.1 Different Pre-Treatments.** A. Standard H&E, post-mortem, 2007, white matter (40X); B. Standard H&E, post-mortem, 2007, cortex (40X); C. Post-fixation Formalin 10%, white matter (40X); D. Post-Fixation Formalin 10%, cortex (40X); E. No pre-treatment, white matter (40X); F. No pre-treatment, cortex (40X); G. Washing Running Water, white matter (40X); H. Washing Running Water, cortex (40X).

#### 4. HISTOLOGICAL METHODS USING MUSEUM SPECIMENS OF THE CENTRAL NERVOUS SYSTEM



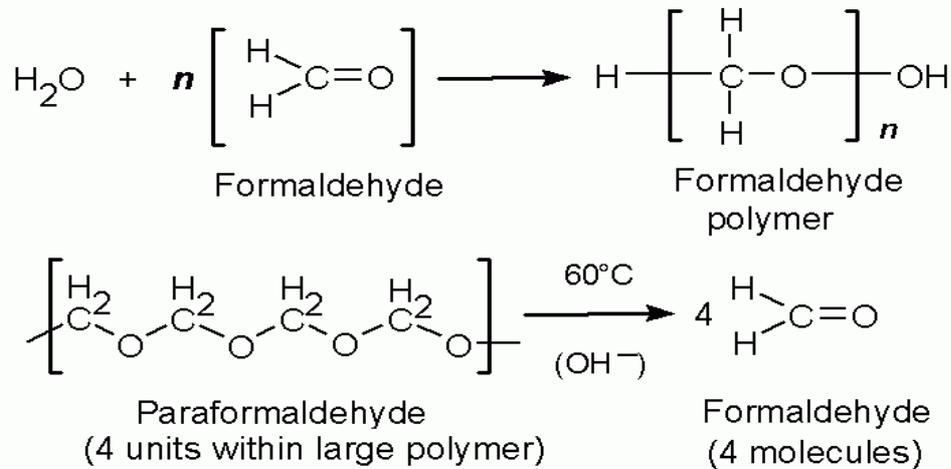
**Fig 4.2 Thickness of the Sections.** A. Standard H&E, post-mortem, 2007, white matter (40X); B. Standard H&E, post-mortem, 2007, cortex (40X); C. 3µm section, white matter (40X); D. 3µm Section, Cortex (40X); E. 5µm Section, white matter (40X); F. 5µm Section, Cortex (40x).

#### 4.4 Discussion/Conclusions:

This study has shown that post-fixation in a 10% formalin solution appears to produce better sections and staining (Fig 4.2) with less evidence of tissue shattering or cracking (Table 4.1) than the other pre-treatments tested. Samples that have been post-fixed in 10% formalin can be compared favourably to more recently sampled tissue of 2007. However, the integrity of nuclear detail, chromatin and nucleoli in the older specimen (1953) is less distinct than more recently fixed samples (2007). In 2000, Barnes and colleagues reported that DNA can be absent in specimens that have been preserved in alcoholic solutions because alcohol does not fix the tissue but only leads to a degree of protein denaturation (Stoddart, 1989). Therefore, the interior of the specimen could contain active nucleases that would degrade DNA, in specimens that have not been properly fixed (Barnes, 2000). The results suggest that prolonged storage may diminish the level of fixation of the specimen, and therefore, samples should be post-fixed in formalin to preserve the original architecture of the tissue. Some possible explanations can be found:

i) The solution loses some of its fixative properties over time:

Formaldehyde is a gas but it can be dissolved in water in a maximum concentration of 37%-40%. When dissolved in water, small molecules of  $\text{-HCHO}$  of formaldehyde will react with  $\text{H}_2\text{O}$  molecules and form methylene hydrate,  $\text{HO-CH}_2\text{-OH}$  (Fig 4) (Le Botlan *et al*, 1983). Methylene hydrate molecules chemically combine with each others to form polymers (Fig 4). Short chain polymers correspond to formaldehyde while higher polymers form paraformaldehyde (Kiernan, 2000).



**Fig 4.3 – Polymerisation and Depolymerisation of Formaldehyde Molecules.** Formation of formaldehyde polymers (above) and depolymerisation of paraformaldehyde (below). (adapted from: Formaldehyde, formalin, paraformaldehyde and glutaraldehyde, 2000).

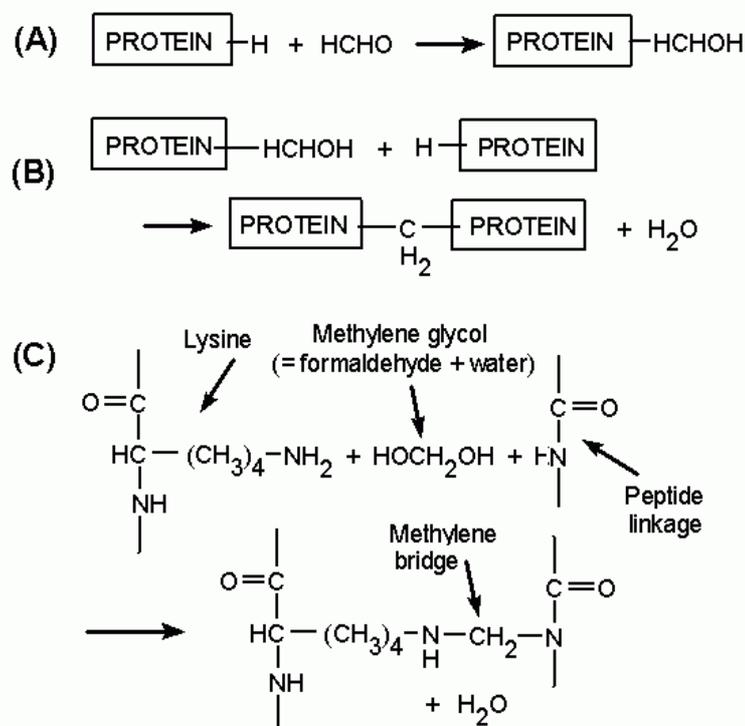
Monomeric molecules of methyl hydrate have to be in a higher concentration so formaldehyde solution can be effective as a fixative (Kiernan, 2000). However the progressive hydrolysis of methyl hydrate polymers, catalysed by hydroxide ions in a slightly alkaline solution will lead to paraformaldehyde precipitation, thus, reducing the fixative strength of the solution.

Formaldehyde molecules also react to produce formate ions. This could be explained by the Cannizzaro reaction (Cannizzaro, 1853): two formaldehyde molecules react together and one is oxidized to formic acid and the other is reduced to methanol. Although this reaction occurs especially slowly, the concentrations of methanol and formate ions in formaldehyde stored for long time are increased (Walker, 1964), reducing formaldehyde molecules in solution.

ii) Formaldehyde bonds with tissue molecules are progressively broken over time:

Formaldehyde forms bonds with tissue proteins. Some studies indicate that the aldehyde group binds to nitrogen atom and some other atoms of proteins. If two such atoms are very close together, the aldehyde group could also form a

cross-link  $-CH_2$  called a methylene bridge (Blum, 1983b). The most reactive sites are primary amines, amide groups, thiols, alcoholic hydroxyl groups and cyclic aromatic rings, (Mason and O'Leary, 1991) and (Fox *et al*, 1985). These types of linkages are assumed to be stable (Yamashita, 2007). However, the subsequent cross-linking of these functional groups to less reactive groups, such as primary amides (glutamine, asparagine), guanidine groups (arginine), and tyrosine ring carbons can be easily reversed by the simple process of washing (Yamashita, 2007).



**Fig 4.4 Reactions Involved in Fixation by Formaldehyde.** (A) Addition of formaldehyde molecule to a protein. (B) Reaction of bound formaldehyde with another protein molecule to form a methylene cross-link. (C) A more detailed depiction of the cross-linking of a lysine side-chain to a peptide nitrogen atom. (Adapted from: Kiernan, J.A., Formaldehyde, formalin, paraformaldehyde and glutaraldehyde, 2000).

This is supported by our results which showed that washing the sample in running water overnight will reverse the fixation effects. The white matter appeared to be more rarefied (Fig. 4.2 G.) even comparing to the sample that had no pre-

treatment (Fig. 4.2 E), which suggest that the museum preservative fluid still preserves some of the fixation cross-linkages.

Molecules as carbohydrates, lipids and nucleic acids are trapped in a matrix of insoluble and cross-linked proteins but are not chemically changed by formaldehyde unless fixation is prolonged. The fixative action of formaldehyde is probably due entirely to its reactions with proteins (Kiernan, 2000) so as long as the cross-links with the proteins are conserved, the other molecules will be preserved as well.

Adequate fixation of tissue is very important in diagnostic practice since artefacts could resemble pathological features. The fixation artefacts showed in picture E and G of the figure 4.2 can be confused with vacuoles, for example. Samples that are not adequately fixed also present an uneven stain (Fig 4.2).

Since the specimen had been fixed for a long time and has already some level of dehydration, the tissue is more dry, brittle and fragile. Treatment with grading alcohol, clearing agents such as xylene, and infiltration with high temperature paraffin could be a very aggressive treatment to tissue in such a state. However, results show that a twelve hour cycle, in some cases, is inadequate (Table 4.1). This issue can be arguable because, processing does not only depend on the properties of the tissue but also in the properties of the processor machine, temperature, use of vacuum and type of organic solvents.

Dryness and brittleness of the tissue can also leads to difficulty in cutting of the tissue on the microtome. Although 5  $\mu\text{m}$  thick sections could be as easily focused at high magnification as the 3 $\mu\text{m}$  thick sections, sections of 5 $\mu$  thick were easier to cut and stained with better quality also (Fig 4.3). Bright stain intensity by eosin was difficult to achieve in the older tissue samples than in more recently fixed tissue. This could be explained by the progressive lowering of the pH of the specimen environment which could slightly change the tissue structures chemical properties. Eosin is an acid stain which binds to eosinophilic structures in the tissue. Although there is a diminished need of using eosin at a correct level of pH of 4.8-5.0 in routine fresh tissue, in tissue preserved for long periods of time this step seems to be essential. The time of eosin staining needs also to be increased.

## **5. IMMUNOHISTOCHEMICAL METHODS USING PATHOLOGICAL MUSEUM SPECIMENS**

## **5. IMMUNOHISTOCHEMICAL METHODS USING PATHOLOGICAL MUSEUM SPECIMENS:**

### **5.1 Introduction:**

Prolonged formaldehyde fixation leads to cross-linking and covalent alterations of protein molecules, giving rise to structural and electrostatic changes of the antigens, thereby reducing antibody immunoreactivity in paraffin sections (Shi *et al.*, 1997). Nevertheless, the use of Epitope Retrieval (ER) techniques can reverse the modification induced by formaldehyde fixation on antigens, thus re-establishing the immunoreactivity (Shi *et al.* 1991, 1993, 1995, 2001, 2002). Such techniques include Heat-Induced Epitope Retrieval (HIER) and Proteolytic Enzymes Epitope Retrieval (PrER) in which tissue sections are heated in a buffer or incubated with proteolytic enzymes respectively, prior to incubation with the primary antibody (Shi *et al.* 1997; Pileri *et al.* 1997; Evers and Uylings, 2000; Boenisch *et al.*, 2001; Kahveci *et al.*, 2003). It is reported that HIER is more efficient than PrER with the majority of antibodies (Pileri *et al.*, 1997). Furthermore, Cattoretti and colleagues (1997) suggested that processing tissue in organic solvents and embedding tissue in molten hot paraffin, in conjunction with the formaldehyde fixation, would also play a role in the diminished immunodetection of antigens in Formaldehyde-Fixed Paraffin-Embedded tissue (FFPE).

This study will investigate the efficacy of immunohistochemistry in FFPE tissue sections. It will also compare immunohistochemistry results obtained in FFPE tissue sections with formaldehyde fixed cryostat sections which did not undergo any processing in organic solvents and paraffin embedding procedures.

### **5.2 Material and Methods:**

Two histological samples (tissue blocks measuring 1x1x1cm) were taken from each specimens: I.A.S. 5883, I.A.S. 5202, E142, E144, I.A.S.4424, I.A.S. 5425, 1987 and 2002 (see Tab 1 – Chapter 2). The samples were washed

overnight in running tap water and then placed in a solution of 10% formalin for 24 hours. Tissue was prepared so that either cryostat or paraffin section could be cut (see below). The antibody selected for this experiment was the monoclonal mouse anti-Human neurofilament protein (Clone 2F11, DakoCytomation) for two main reasons: a) neurofilament protein is ubiquitously expressed in the central nervous system (Diepholder *et al*, 1991; Luidere *et al*, 1992), and b) neurofilament protein appears to be more resistant to prolonged fixation as compared to other neuromarkers, even in tissue stored up to ten years (Lick *et al.*, 2008).

### Formalin fixed cryostat sections:

Following post-fixation in 10% formalin, one tissue blocks of each specimen was cryoprotected in 20% sucrose for 48 hours at 4°C. The tissue samples were then snap-frozen in liquid nitrogen, and 10µm cryostat sections were cut. The sections were maintained at 4°C for 72 hours. They were then left to dry at room temperature for three hours prior to the immunostaining protocol (see below).

### Formalin fixed paraffin sections:

Following post-fixation in 10% formalin, one tissue blocks of each specimen was processed to paraffin using a 24 hour cycle using a previously established protocol (Chapter 3). The tissue was then embedded in paraffin and 5µm sections were cut (see Chapter 3 for more detail). The sections were deparaffinised in xylene (2 x 3 minutes), re-hydrated (absolute ethanol 3 minutes, methanol 3 minutes, 70% methanol 3 minutes and tap water minutes) and finally immersed in Tris NaCl Tween (TNT) buffer (0.025M Tris pH 7.5, 0.03M NaCl and 0.05% Tween-20) for 3 minutes. The sections underwent microwave (Samsung Selection 800W) antigen retrieval: Tris Ethylene Glycol (TEG) buffer (10mM Tris pH 9.0 and 0.5mM Ethylene Glycol Tetraacetic Acid (EGTA) for 9 minutes at 850W (Lick *et al.*, 2008). The sections were allowed to cool down to room temperature for approximately 20 minutes prior to immunostaining.

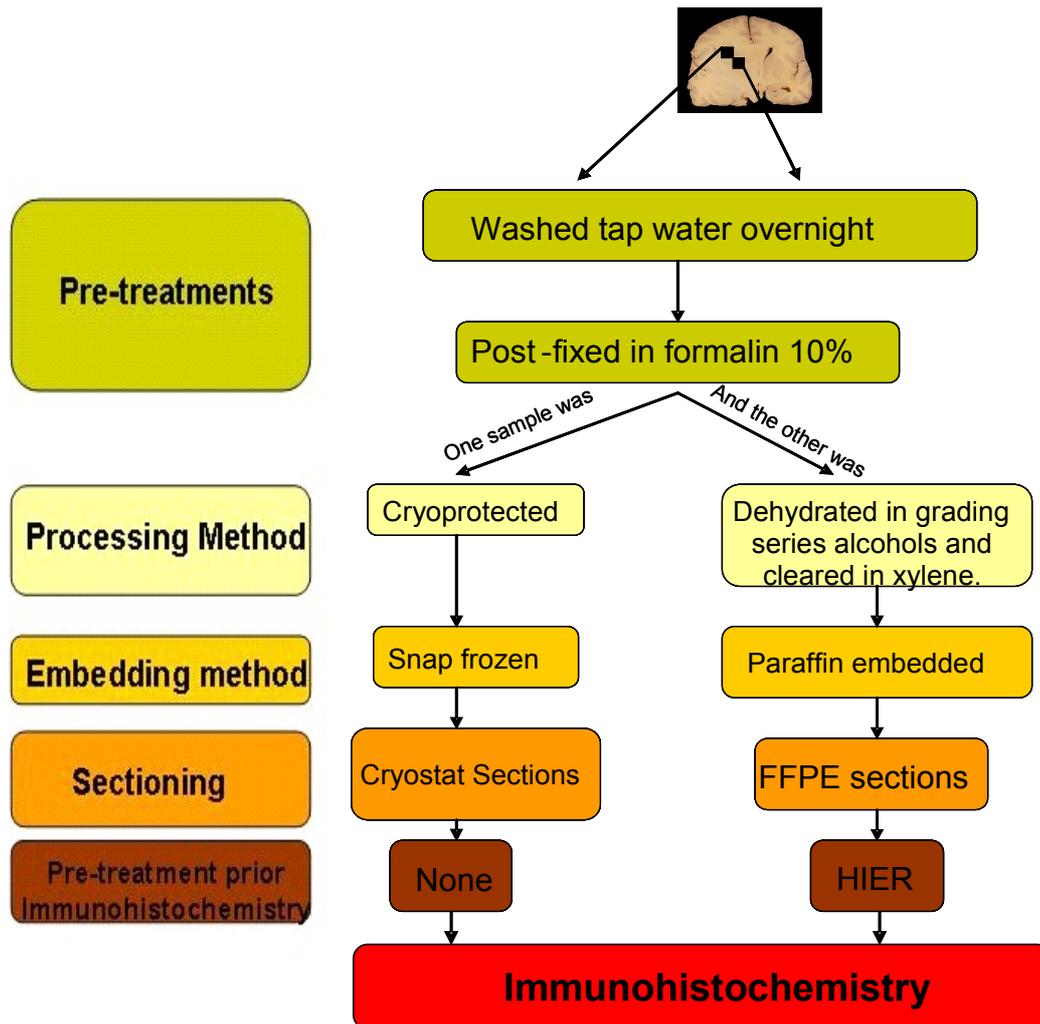
One extra section of the I.A.S. 5883 specimen block and another one from the block of the specimen of 2007 (see Table 1, Chapter 2) were used as a negative and positive control, respectively.

Immunohistochemistry:

All incubations were carried out in a humidity chamber.

Sections were washed in TNT buffer for 5 minutes. Endogenous peroxidase activity was eliminated by incubating the sections for 5 minutes in DakoCytomation EnVision+ System-HRP Peroxidase Block. The sections were rinsed in TNT buffer for 5 minutes, and incubated for 7 minutes at room temperature in Protein Block Serum-Free (DakoCytomation) to eliminate non-specific staining. The sections were then incubated with the primary antibody – monoclonal mouse anti-human neurofilament protein (Clone 2F11, DakoCytomation) for 30 minutes using a dilution of 1:200. TNT buffer was used in the negative control section instead of the primary antibody. The sections were rinsed in TNT buffer for 5 minutes, and then incubated with the secondary antibody, the peroxidase labeled polymer (DakoCytomation EnVision+ System-HRP) for 30 minutes. The sections were rinsed TNT buffer for a further 5 minutes. Finally, the sections were incubated in the substrate-chromogen (DakoCytomation EnVision+ System-HRP) for 6 minutes, and then rinsed again in TNT buffer for 5 minutes.

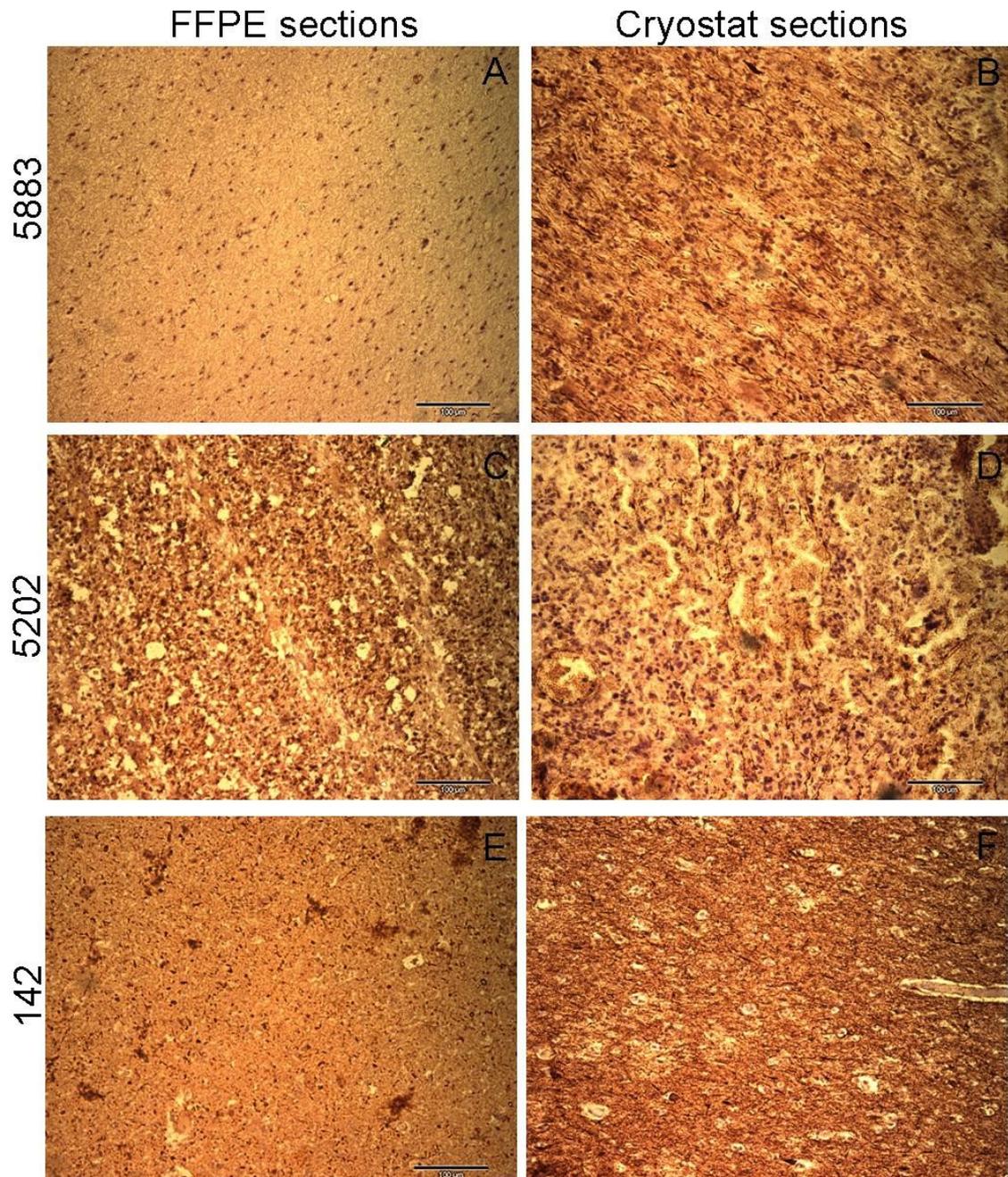
Sections were counterstained in Ehrlich haematoxylin for 3 minutes, immersed in running tap-water for bluing for 3 minutes, differentiated in acid-alcohol, and returned to running tap water for 3 minutes for bluing again, dehydrated (70% methanol 3 minutes, methanol 3 minutes and 100% ethanol 100%) and cleared (2 x 3 minutes xylene). The sections were mounted in DPX medium and cover-slipped.

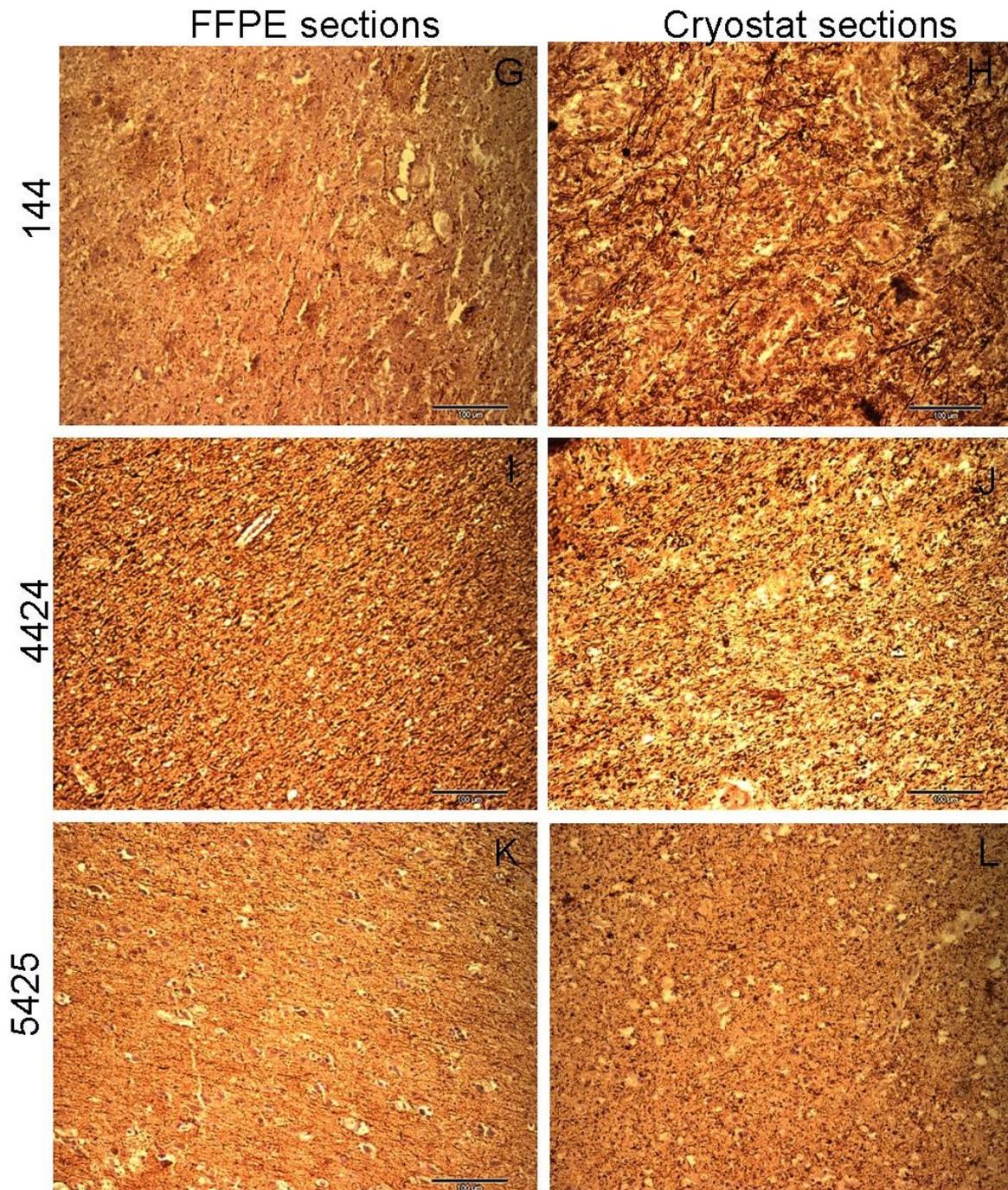


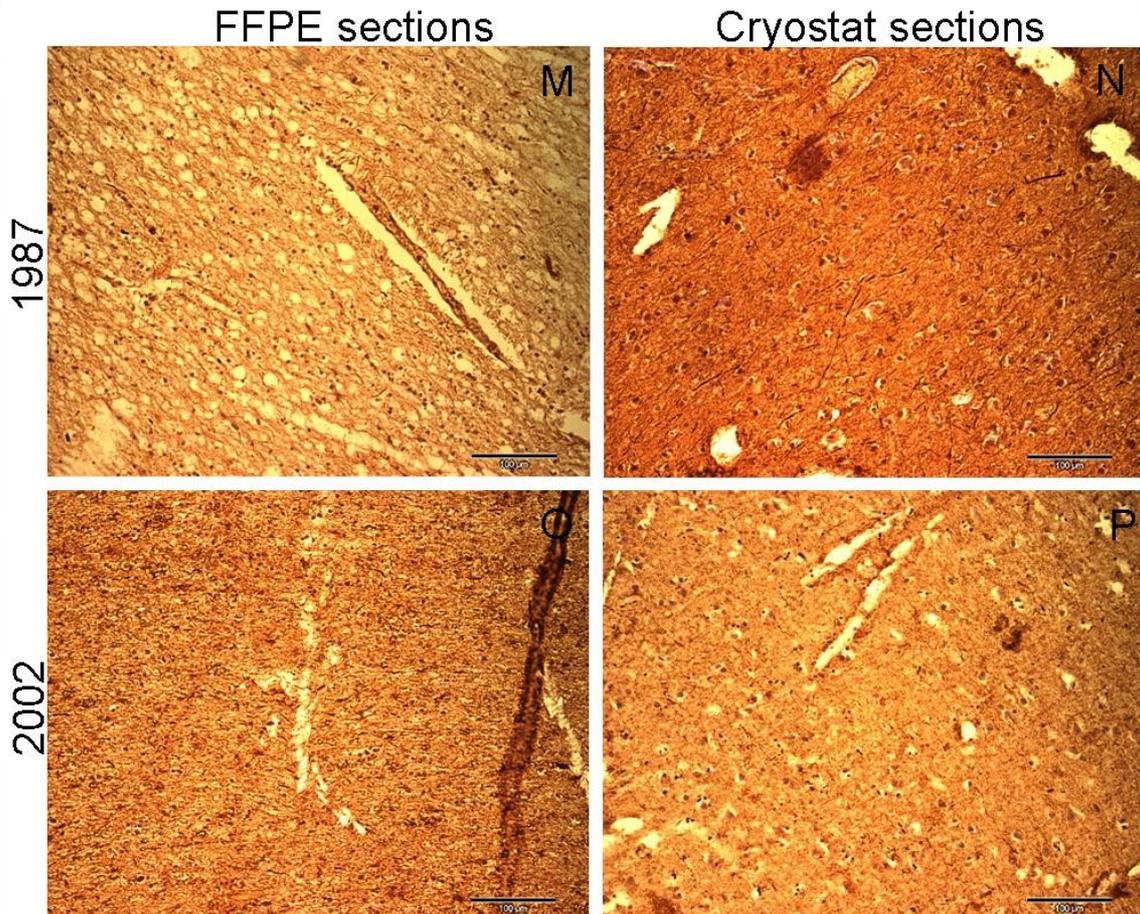
**Fig.5.1 – Flow Chart Describing the Experimental Method.** Samples of each specimen underwent all the steps described in this flowchart.

### 5.3 Results:

The microphotographs of the immunostained sections for anti-neurofilament of the each specimen are shown below.







**Fig 5.2 – Anti-Neurofilament Protein Immunostaining.** Comparison between immunostaining in cryostat sections and formalin-fixed paraffin-embedded (FFPE) sections. **A.** Specimen I.A.S. 5883, FFPE section. **B.** Specimen I.A.S. 5883, cryostat section. **C.** Specimen I.A.S. 5202, FFPE section. **D.** I.A.S. 5202, cryostat section. **E.** Specimen E142, FFPE section. **F.** Specimen E142, cryostat section. **G.** Specimen E 144 FFPE section. **H.** Specimen E 144, cryostat section. **I.** Specimen I.A.S. 4424, FFPE section. **J.** Specimen I.A.S. 4424, cryostat section. **K.** Specimen I.A.S. 5425, FFPE section. **L.** Specimen I.A.S. 5425, cryostat section. **M.** Specimen 1987, FFPE section. **N.** Specimen 1987, cryostat section. **O.** Specimen 2002, FFPE section. **P.** Specimen 2002, cryostat section.

In the specimens I.A.S. 5883, I.A.S 5202, E142, E144 and 1987, cryostat sections had produced a specific and more intense staining comparing to FFPE.

The neurofilament present in neurons processes is clearly stained in the white matter and it is surrounded by glial cells (not immunostained). Immunostaining of FFPE in these specimens was very faint and in the specimen I.A.S. 5883 there was no immunoreactivity at all. However, in sections of the specimens I.A.S. 5425 and 2002, FFPE sections had shown more intensity of the staining comparing to cryostat sections. Section of the specimen I.A.S. 4424 had shown immunostaining deposits, hampering microscopic observation.

### **5.4 Discussion/Conclusions:**

Although there are studies that documented the success on retrieving immunoreactivity of neurofilament in tissue preserved up to ten years (Lick *et al*, 2008), this preliminary study indicates that it appears to be possible to perform immunohistochemistry staining of human neurofilament protein even in tissue from specimens stored up to fifty-five years in museum preservative fluid (Kaiserling solution) (Fig 5.2).

Another important fact to highlight is that, initially it was thought that a higher concentration of the antibody was required due to the supposition that the immunoreactivity would be decreased. This initial supposition proved to be false because although the recommend dilution of the anti-neurofilament antibody (DakoCytomation) was ranging between 1:200 to 1:50, some initial tests prior the experiment showed that the lowest concentration (1:200) had more intense and specific staining with no background (Fig 5.2).

These results can be even more interesting because a monoclonal antibody (Kohler and Milstein, 1975) was used. Monoclonal antibodies can be more challenging because they are unique molecules that recognize only a single epitope of the antigen which demonstrates that antigens are well preserved and a very specific immunostaining is possible (Fig 5.2).

This study suggest that formalin fixed cryostat sections produce a more intense and specific immunostaining than FFPE sections (Fig 5.2). Although it is widely known that formaldehyde fixation mask antigens by mean of cross-linkages between proteins, thus reducing the detection of antigens, this can not be a coherent explanation since both samples were taken from the same specimen, from the same region and fixed exactly for the same numbers of years in the same preservation conditions.

Furthermore, HIAR was performed in the FFPE because accordingly to some publications (Shi *et al.* 1991, 1993) the antigen masking caused by formaldehyde fixation can be reversed with this pretreatment. HIER methods can cleave protein-protein crosslinks, by two major factors: a) temperature, and b) pH.

In 1991, Shi and colleagues demonstrated that heating effectively increases immunostaining of many antigens in FFPE sections. In HIER techniques, the heating cleaves the crosslinks and methylol groups are added to proteins. Heating also extend polypeptides chains, thus exposing the entangled epitopes in the inner portion of antigens. In 2005, Yamashita and Okada demonstrated that heating does cleave the crosslinks caused by formaldehyde. They purified 5 proteins and exposed them to 4% formaldehyde. Then, heating was applied and they were run in a gel and analysed by SDS-PAGE. Apparently the proteins that have been treated after fixation yielded almost the same results as unmodified proteins.

The pH of the buffer solution in the HIER also plays a role in ER as it avoids the re-masking of the antigens after the microwave heating during the cooling process. Some studies concluded that solutions at acidic or basic pH values are more suitable for HIAR (Shi *et al.* 1993, 1995; Evers and Uyling, 1997; Ferrier *et al.*, 1998, Brorson, 2002; Kim *et al.*, 2004, Yamashita and Okada, 2005; Emoto *et al.*, 2005), however, acidic environment proved to be too aggressive to tissue integrity. Random entangling of polypeptides while cooling down, after HIER, seems to occur when using buffer with pH values ranging 4.5-7.5. Apparently, hydrophobic and electrostatic forces act in cooperation and polypeptides entangles, thus concealing epitopes (Yamashita, 2007).

The results of this study indicate that the unmasking of the antigen by HIER is not complete because immunoreactivity of neurofilament in FFPE was very low

(Fig 5.2) even after performing the epitope retrieval technique that was considered the one that produces better results in long-term storage tissue for immunostaining of neurofilament (TEG buffer for 9 minutes in the microwave) (Lick *et al*, 2008).

This raised the idea that possibly the antigen is not only masked during fixation by formaldehyde. Although the specimens 5883 and 5202 were fixed for at least 55 and 54 years respectively, cryostat sections produced good level of immunostaining for neurofilament (Fig 5.2), this can be explained by either the fact that Kaiserling solution has no formaldehyde in its composition, and because museum specimens are firstly fixed in formaldehyde and then stored in a formaldehyde-free preservative solution, antigens epitopes are more easy to retrieve than those from archival specimens that have been fixed in formaldehyde for years, or because the formaldehyde fixation is not the major reason for masking antigens.

It is of note that immunostaining for neurofilament in cryostat sections is more optimal than that observed in FFPE sections (Fig 5.2). This may be related to the different processing protocols employed for each of the samples. The only difference was that cryostat sections were cryopreserved and snap-frozen whereas the FFPE were processed in grading alcohols and embedded in molten paraffin (+60°C). The results presented in this chapter support the studies of Cattoretti and his colleagues in 1993 which indicated that processing with organic solvents and the heating involved in the paraffin embedding procedure also masks epitopes by changing conformational shape of molecules.

## **6. INVESTIGATING MUSEUM SPECIMENS OF NEUROLOGICAL DISEASE**

## **6. INVESTIGATING MUSEUM SPECIMENS OF NEUROLOGICAL DISEASE:**

### **6.1 Introduction:**

Previous chapters have described methods to optimise the processing of archival museum tissue, and establish protocols for cutting and staining tissue sections. The aim of this chapter is to carry out a preliminary study to establish the feasibility of making histological diagnoses in archival museum specimens of macroscopically defined neurological disease.

The three oldest specimens (I.A.S. 5883, I.A.S. 5339 and I.A.S. 5202) were selected for case analysis using histology and immunohistochemistry staining. The reason why the three selected cases are the oldest can be explained based in the working hypothesis that the levels of tissue preservation and antigen integrity decreases with age. Therefore, it is believed that if diagnostically good results can be achieved with the oldest, possibly the same or even better results can occur in the more recent cases.

### **6.2 Material and Methods:**

For histological analysis, sections were cut from the paraffin blocks already prepared in Chapter 5. Each section was deparaffinised (xylene for 2X5minutes) and hydrated (methanol 70% for 5 minutes, methanol 100% for 5 minutes, ethanol 100% for 5 minutes and tap water for 5 minutes). They were stained in Ehrlich haematoxylin for 5 minutes, were blued in running tap water for 5 minutes, differentiated in acid-alcohol (two dips) and placed in running tap water for 5 minutes to stop the differentiation. Sections were stained in eosin for 3 minutes (with adjusted pH 4.8-5.0), dehydrated (methanol 70% for 5 minutes, methanol 100% for 5 minutes and ethanol 100% for 5 minutes) and cleared (xylene X 2 for 5

minutes each). Sections were mounted using resin medium (DPX) and coverslipped.

The sample I.A.S. 5883 was prepared for immunohistochemistry staining, and therefore a new block had to be cut for processing for cryostat sections. The block was cut from the lesion area (1cmX1cmX1cm), was washed overnight in running tap water and then placed in a solution of formalin 10% for 24 hours. Then, it was prepared for cryostat sections (see below):

#### Cryostat Sections:

Sample was cryoprotected in a solution of sucrose 20% for 48 hours in the fridge at 4 °C. It was snap-frozen in liquid nitrogen and sectioned in the cryostat at 10 µm thick. The section was air-dried for 3 hours prior to immunostaining.

#### Immunohistochemistry:

A section from specimen I.A.S. 5883 was immunostained with polyclonal rabbit Anti-Glial Fibrillary Acidic Protein (1:1200, DakoCytomation) using ABC method (VECTASTAIN® Elite ABC Kit PK-6200). All incubations were made using a humidity chamber. The section was hydrated in tap water for 5 minutes and balanced in phosphate buffered saline (PBS) for 5 minutes. Then, quenching of endogenous peroxidase was made placing the section in a bath of 3 % hydrogen peroxide for 20 minutes. The section was rinsed in PBS buffer for 5 minutes, and blocked with normal serum (ABC kit) for 20 minutes. The normal serum was tapped off and the section was incubated in primary antibody anti-GFAP in a concentration of 1:1200 overnight. The slide was washed in PBS for 5 minutes and incubated in the universal biotinylated secondary antibody for 20 minutes. The section was rinsed in PBS buffer for 5 minutes and incubated for 20minutes with Vectalite ABC complex (SS label) that was previously prepared according to manufacturers recommendations. The section was rinsed in PBS buffer for 5 minutes. The staining was the developed using DAB chromogen (Vector Laboratories) for 10 minutes. Then section was washed for 10 minutes in PBS buffer and immersed in a bath of copper sulphate solution (0.5% CuSO<sub>4</sub> in dH<sub>2</sub>O) for 4 minutes and washed in tap water for 5 minutes. The section was

counterstained using Gills II haematoxylin for 10 seconds, washed in running tap water for 10 minutes, dehydrated (alcohol 95% 2x5 minutes, alcohol 100% X 2 for 5 minutes) and cleared in xylene for 2x5minutes. Section was mounted in DPX and coverslipped.

### **6.3 Results:**

#### **6.3.1 I.A.S. 5883:**

##### **6.3.1.1 Macroscopic findings:**

The pot was labelled "cystic astrocytoma". Examination of a coronal brain slice at the level of mammillary bodies reveals a diffuse lesion in white matter, above the basal ganglia, in the right hemisphere of the temporal lobe (Fig 6.1 A).

Note the substantial mass effect and the diffuse character of the tumor. The tumour mass is also compressing the left lateral ventricle. There is blurring of the gray-white matter junction. Several and small areas of cavitation can be seen (Fig 6.1 B).

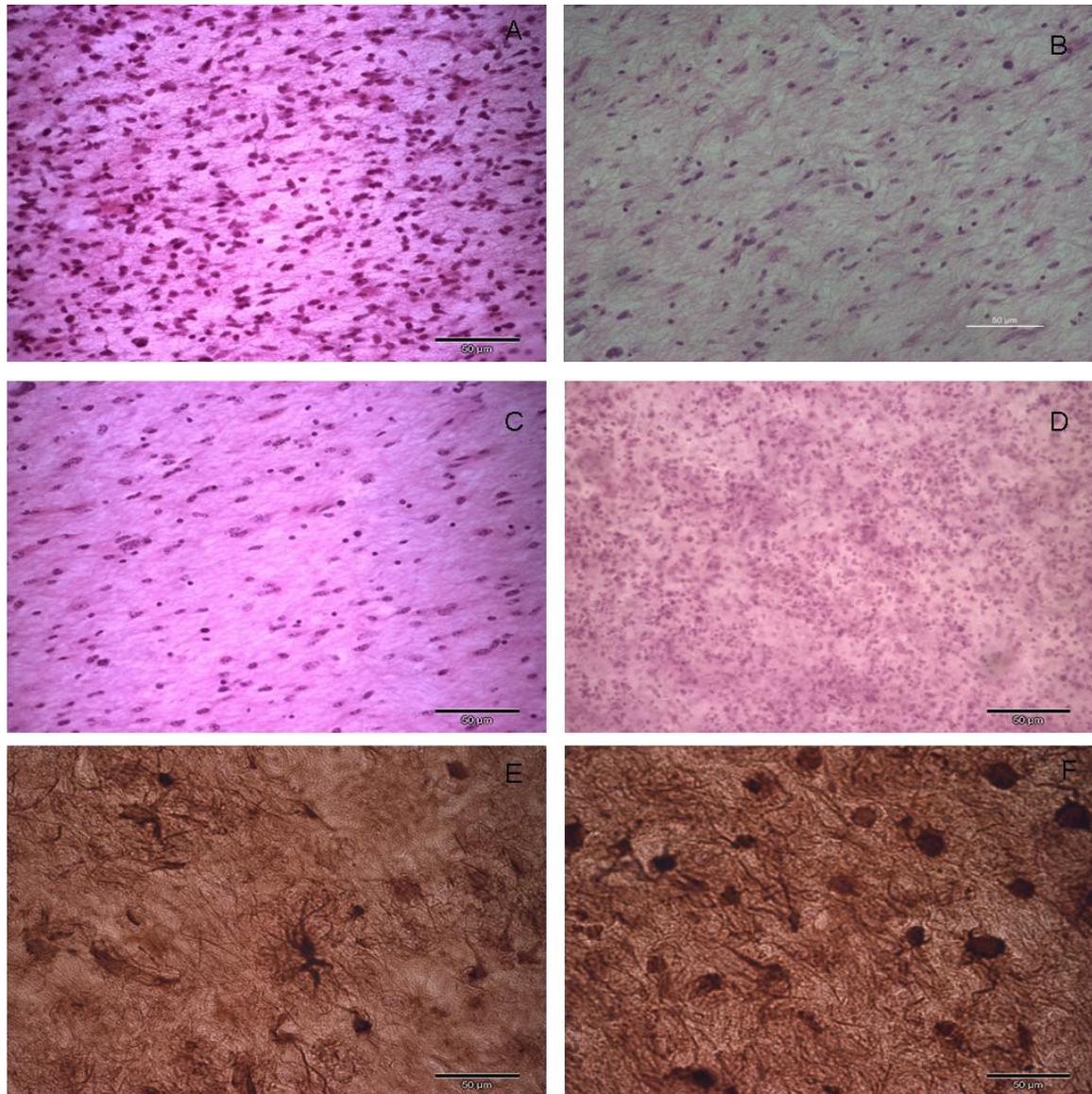
The sampling for histological specimens was performed on the reverse side of the brain slice to preserve the display properties of the specimen in the pathology museum (Fig 6.1 C). This happened only in the cases that the lesion crossed all the depth of the specimen and the sample was considered representative of the lesion.



**Fig. 6.1 – Macroscopic Picture of Specimen I.A.S. 5883 (1953).** A. Coronal section of the brain – diffuse lesion in the right hemisphere. B. Close up picture of the lesion. C. Location of the sampling for histology.

### **6.3.1.2 Microscopic Findings:**

The section shows the histological features of an astrocytoma. The tumour is hypercellular and consists of astrocytic cells with enlarged pleomorphic nuclei (Fig 6.2 – A, B and C). The tumour infiltrates the adjacent brain parenchyma (Fig 6.2 - B and C). Immunohistochemistry reveals a number of GFAP immunopositive tumour cells (Fig 6.2 - E and F). It is of note that while immunohistochemistry in cryostat sections produces more optimal results, this is at the expense of tissue preservation which makes the HE stained cryostat sections more difficult to interpret than equivalent paraffin embedded sections (Fig 6.2 – D).



**Fig 6.2 Histological and Immunohistochemical Staining of Sections from Specimen I.A.S. 5883 (1953).** A. FFPE section showing changed white matter. H&E. 40x B. FFPE section showing changed white matter. H&E. 40x. C. FFPE section showing altered white matter. H&E. 40x. D. Cryostat section showing altered white matter. H&E. 40x. E. Cryostat section immunostained for anti-GFAP. 40x. F. Cryostat section immunostained for anti-GFAP.40x.

### **6.3.1.3 Discussion/Conclusions:**

For histological analysis the optimised protocol was used (Chapter 4) and it was adequate for diagnostic purposes.

Cryostat sections were used for immunohistochemistry as they had shown better immunoreactivity compared with FFPE (Chapter 5). However, when stained with H&E it demonstrates poor preservation of the original tissue architecture. This was not expected as the tissue was fixed and, moreover, had undergone post-fixation in formaldehyde 10% for 24 hours. Nonetheless, pre-treatment with sucrose 20% for 48 hours may have removed some of the formaldehyde cross-linkages in the tissue, thus reversing fixation effects. In future studies using fixed tissue processed for cryostat sections, a post-fixation in an acetone solution will be attempted.

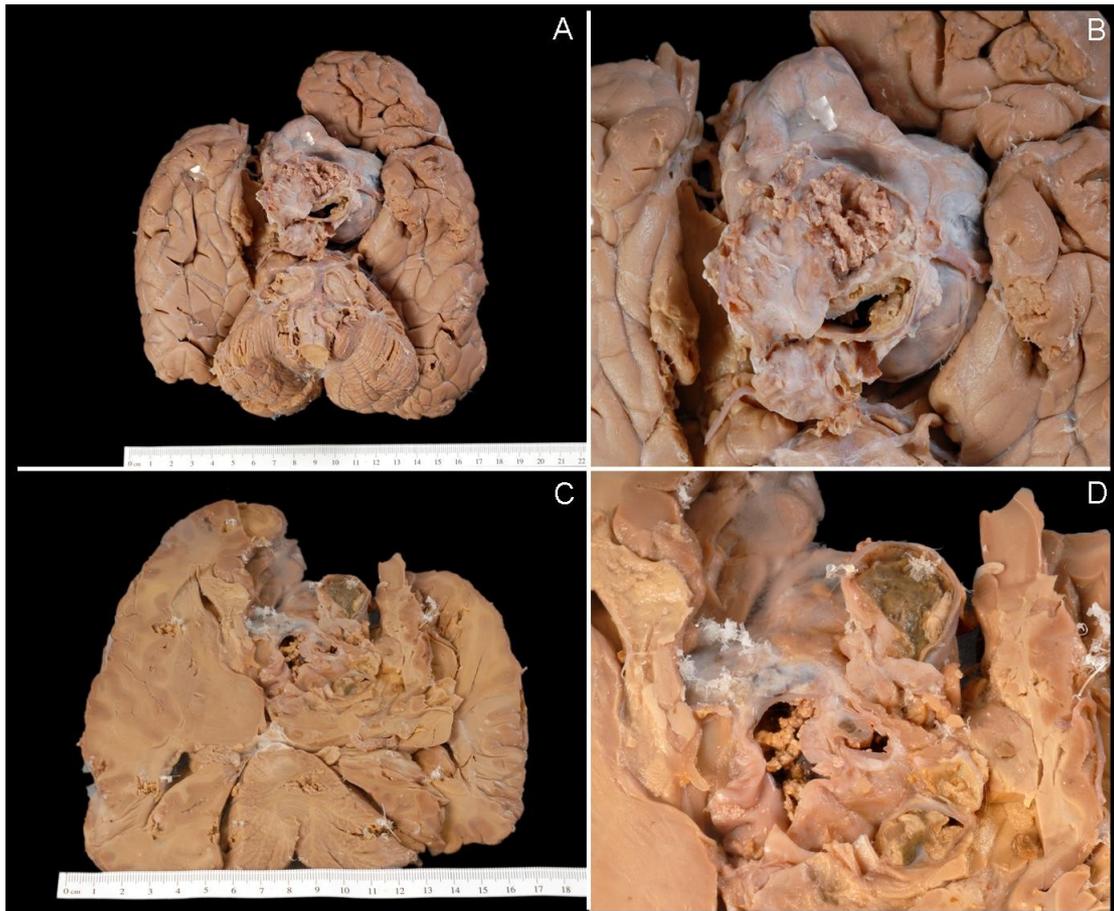
A different method was used, the ABC method, because there was an interest in knowing if a different method would yield even better results. The immunohistochemistry staining showed the presence of background staining. This could be explained by several factors: a) the length of incubation time with primary antibody was too long; b) the concentration of the antibody was too high; c) the length of incubation time in DAB was too long. These results showed that the immunohistochemistry protocol used in Chapter 5 apparently produces better results, and some alterations to the protocol used with ABC method need to be done in order to yield more satisfactory results when attempting it in museum specimen tissue preserved for decades.

### **6.3.2 I.A.S. 5339**

#### **6.3.2.1 Macroscopic findings:**

The specimen I.A.S. 5339 was labelled with two possible differential diagnoses: a) "Giant Aneurysm" b) "Craniopharyngioma".

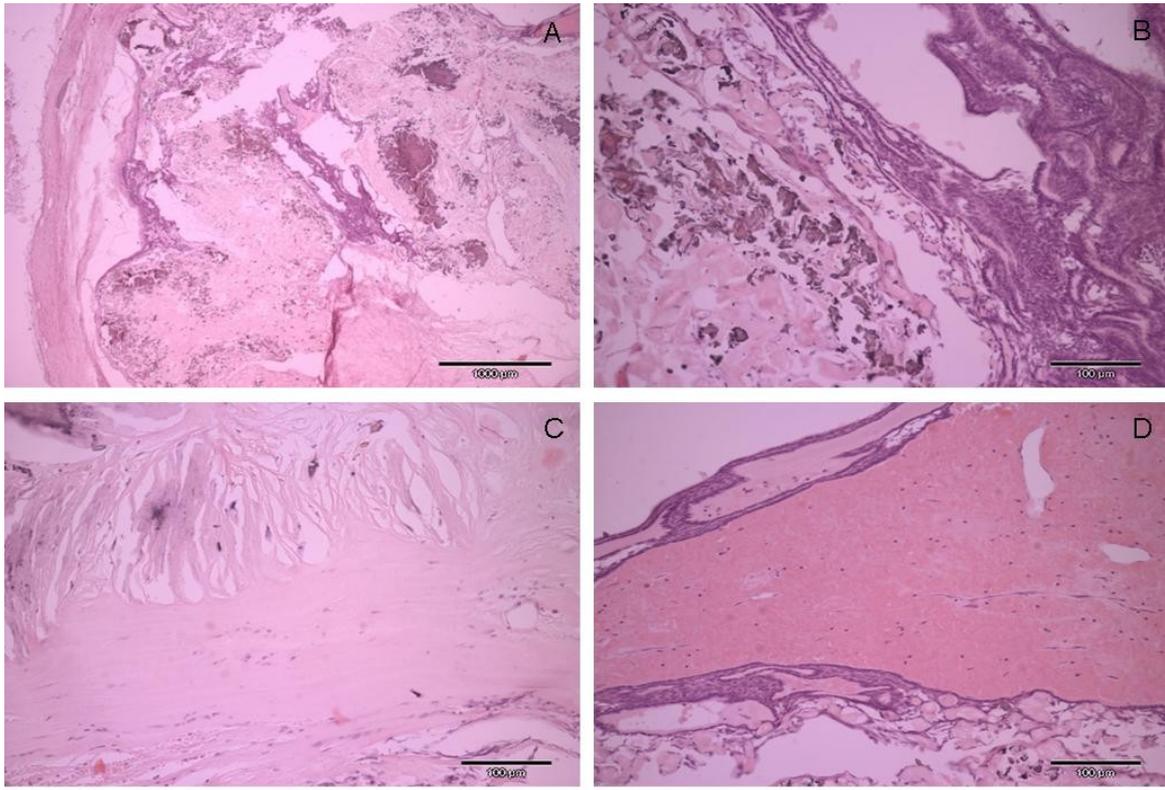
The examination of the base of the brain reveals a lesion consisting on a large round mass, measuring 6x7cm, located in the suprasellar region (Fig 6.3 A). The mass is cystic and surrounded by a membrane and has an irregular rounded shape (fig 6.3 B). A horizontal section of the brain show that the mass is connected to brain and it is compressing some structures (Fig 6.3 C). A close up picture of the lesion reveals a heterogeneous and greenish content, with some calcifications (Fig 6.3 D).



**Fig 6.3 Macroscopic Picture of Specimen I.A.S. 5339 (1954).** A. Lesion in the base of the brain. B. Close-up of the lesion. C. Reverse side of the specimen, horizontal section of the brain. D. Close-up of the reverse side of the specimen. Note the contents of the lesion.

### **6.3.2.2 Microscopic Findings:**

The histological features are those of a craniopharyngioma. Photomicrograph at a low magnification shows fibrotic tissue surrounding heterogeneous content (Fig 6.4 A). The tumor shows the presence of linear epithelium in palisade (Fig 6.4 B). The tumor did contain cholesterol as it is shown by cholesterol clefts (Fig 6.4 C). Haemorrhagic areas can also be seen in this tumor (Fig 6.4 D).



**6.4 Histological Slide of the Specimen I.A.S. 5339 (1954).** A. Low-magnification photomicrograph showing overall appearance. H&E. 2.5x B. Linear epithelium in palisade.H&E. 20x. C. Cholesterol Clefts. H&E. 20x. D. Haemorrhagic area. H&E. 20x.

### **6.3.2.3 Discussion/Conclusions:**

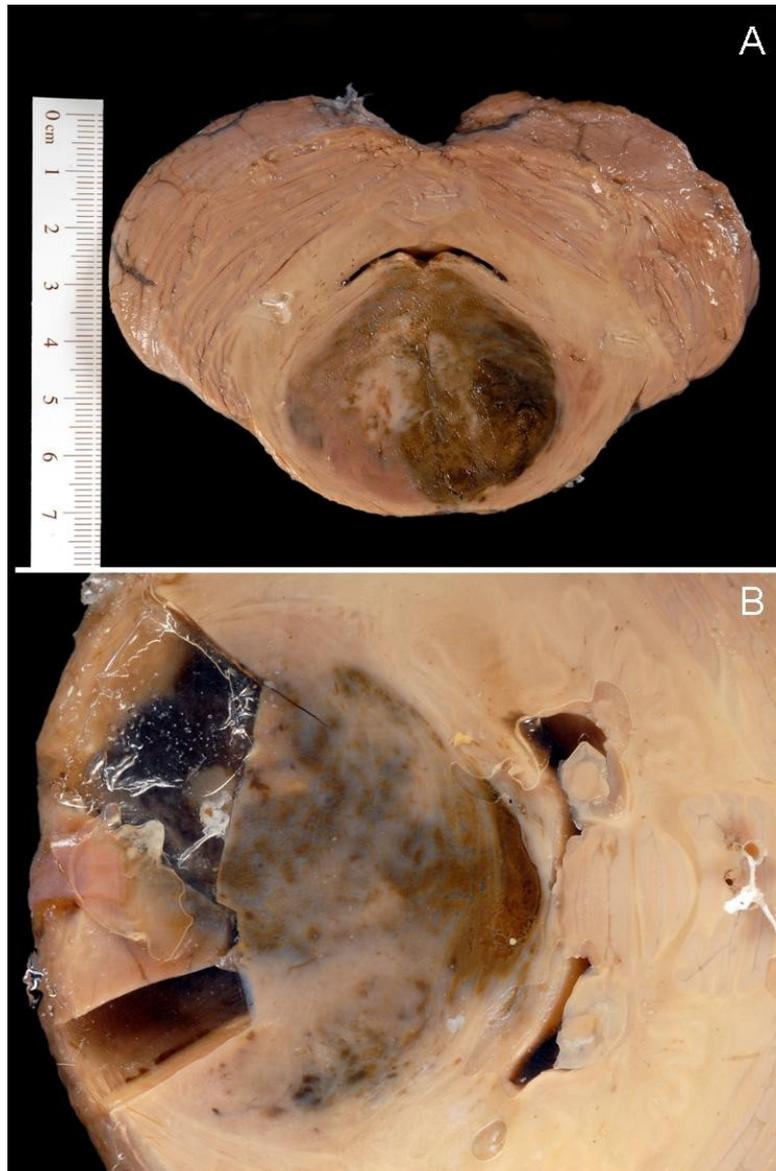
The macroscopic observation of the specimen I.A.S. 5339 reveals a large tumor-like mass with hard consistency, in the suprasellar region on the base of the brain. The histological preparation shows a complex organization surrounded by fibrotic tissue (Fig 6.4 A) and containing keratin nodules, calcification (Fig.6.4 A and B), haemorrhagic zones and inflammatory reaction (Fig.6.4 D). It was also demonstrated by H&E staining features as the palisading of the epithelium (Fig 6.4 B and D) and also cholesterol clefts (Fig.6.4 C). These are all distinctive features of a craniopharyngioma. As the histological preparation stained for H&E was of good quality and clear on the pathological condition, no further immunohistochemistry investigation was required.

### **6.3.3 I.A.S. 5202**

#### **6.3.3.1 Macroscopic Features:**

The specimen I.A.S. 5202 was labeled as a “Brain stem glioma.”

Macroscopic examination shows a large, rounded haemorrhagic lesion, measuring 4x4.5cm in the brain stem at the level of the pons (Fig 6.5 A). Fig 6.5 B shows the location of the histological sampling. Note the shining material covering the specimen. Gelatin is used for covering museum specimens to prevent those parts of the specimens from becoming de-attached, thus decreasing the levels of display properties quality. The four surfaces of the sample block were trimmed prior to histological processing to remove all gelatin.

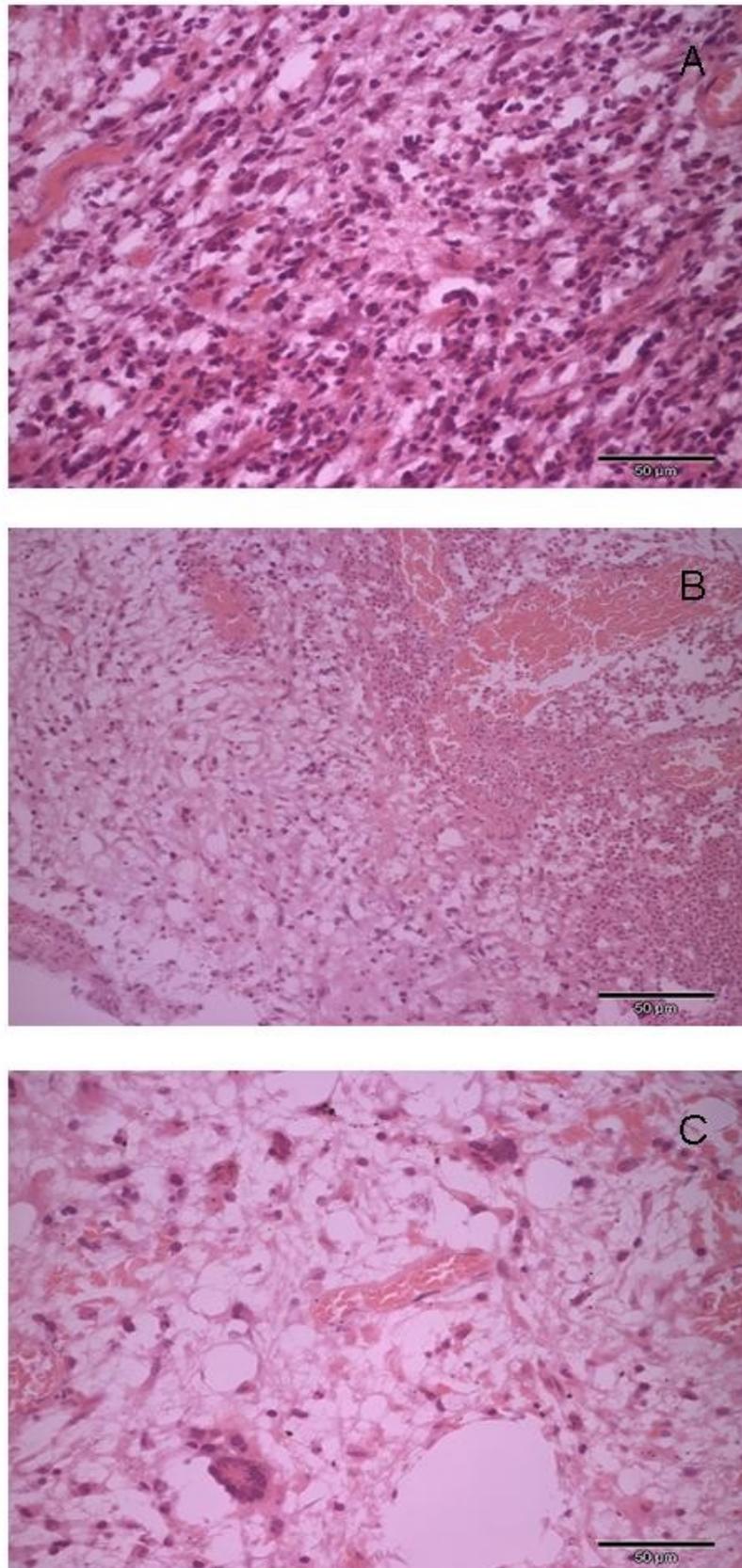


**Fig 6.5 Macroscopic Picture of I.A.S. 5202 Specimen (1955).** A. Horizontal section of brain stem at the level of pons. B. Location of sampling for Histology

### **6.3.3.2 Microscopic Findings:**

The specimen I.A.S. shows features of an astrocytic tumor. This tumor shows high cellularity content (Fig 6.6 A). A haemorrhagic area with inflammatory exsudate can also be seen (Fig 6.6 B). Figure 6.6 C shows clusters of cells.

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**Fig 6.6 Histological Slide of Specimen I.A.S. 5202 (1955).** A. Cellularity content increased. H&E. 40x. B. Inflammatory exudate. H&E. 40x. C. Clusters of cells. H&E. 40x.

### **6.3.3.3 Discussion/Conclusions:**

Specimen I.A.S. 5202 exhibits a haemorrhagic lesion in the brain stem at the level of the pons (Fig 6.5). This well delimited lesion appears to be a brain stem neoplasm.

Histological reveals a focally haemorrhagic glial tumor, probably astrocytic in origin (Fig 6.6 A and B). There also appears to be clusters of cells with a more typically neuronal morphology, but it was not possible to characterize these further (Fig 6.6 C).

The macroscopic and microscopic features suggest that this tumor is an astrocytic tumor, with additional neuronal component. Further characterization using immunohistochemical techniques would be of interest.

## **7. OVERVIEW**

## 7. OVERVIEW:

The study of the properties of museum specimens has shown that it is essential to have an extensive knowledge of the history of the specimen preservation.

1. Knowledge of the age of the specimen and type of mounting fluid is essential in order to control variables that may affect results of further histological techniques. The history of individual pots can be complex and may involve more than one mounting fluid. Chemical composition of mounting fluids is very variable and seems to depend on age of the pot in relation to a particular curator. When the pH of various mounting solutions was compared no difference was found in the means (although it is stressed that for some solution types the sample size is small and definitive conclusions are not yet possible). Interestingly, within the aldehyde group (n = 9) there was a wide range of different pH values (pH 4.19 to 7.48). This indicates that knowledge of the mounting solution cannot be used to accurately predict the pH. This study had shown that pH is correlated with number of years that the specimen had been stored. However, interpretation of this finding is complicated by the fact that particular mounting solutions are not distributed evenly across the age range. The study needs to be extended to a larger sample of each mounting fluid, in order to validate this correlation between pH and years of storage.

2. For attempting histological and immunohistochemical techniques it is important that the samples are washed in running water for the removal of the mounting fluid prior to processing. However prolonged submersion in water is detrimental to preservation of the morphology.

3. Post-fixation in 10% formaldehyde is also required for the preservation of the tissue morphology.

4. It is not possible to standardize the protocol for processing the samples, as this depends on specific features of the sample size and processor machine. The important variables appear to be vacuum, temperature and agitation.

5. Salvage material tends to be brittle and difficult to section on the microtome. But, although it was very difficult to cut sections at 3µm, sections of 5µm were cut with success.

6. In order to optimise the H&E staining protocol it is necessary to bring the pH of the eosin solution to the values in interval ranging 4.8-5.0.

7. Initial impressions are that cryostat processing may be a better approach than paraffin processing when preparing specimens for immunohistochemistry. The immunostaining appears to be more intense and specific in cryostat sections comparing with FFPE sections. The results of this experiment also support the idea raised by previous studies (Cattoretti *et al*, 1993) that suggested that the processing in organic solvents and the embedding in hot molten paraffin alters the conformation of the antigen, entangling epitopes and, therefore, hampering immunohistochemical detection. However, further investigation is required in this area. This can be a very interesting fact considering the increasing number of tissue banks. In some of these banks, tissue is preserved in paraffin blocks. However, as this dissertation shows, freezing samples, or preserving them in Kaiserling solution after formaldehyde fixation, is probably the best preservation method if further immunohistochemistry will be attempted.

8. However paraffin sections seem to preserve better the original architecture of the tissue producing better H&E staining when compared to cryostat sections.

9. Investigations carried out in three pathological specimens using histological and immunohistochemistry preparations pointed out that it was possible to use material that is at least 55 years old to diagnose neuropathological conditions.

## **8. FUTURES STUDIES**

## 8. FUTURE STUDIES:

Some of the results of this dissertation had exceeded the initial expectations, and because museum specimens study, using biomolecular techniques is a field with such a lack of published work, futures studies will be carried out to investigate this.

First of all, experiments will be repeated with more specimens to give a strong consistency to the results and more scientific validity of the conclusions.

Then, the mechanism underlying alteration of antigen conformation will be study in more depth and more immunohistochemical methods will be tested, in order to optimize the immunohistochemical protocol for museum specimens study.

It is also aimed to extend this study to other molecules as DNA or RNA. It has been reported some successful works on extraction of DNA and RNA with optimised protocols (Coombs *et al*, 1999). However, there is awareness that DNA and RNA molecules can be degraded hampering a high quality extraction of nucleic acids, and, moreover, studies on museum specimens made by Barnes and his colleagues in 2000 had shown that DNA and RNA in specimens preserved in alcohol is most of the times degraded, therefore limiting the recovery of DNA fragments.

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## 9. REFERENCES

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