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# The use of a cerebral perfusion and immersion–fixation process for subsequent white matter dissection

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## HIGHLIGHTS

- We describe a technique for minimizing the limitations of Klingler's method.
- Intra-carotideal formalin perfusion fixation of 10 human hemispheres was performed.
- A shorter and more homogeneous fixation provided a high quality of specimens.
- Fiber organization (superficial/deep) and its vascular architecture were described.
- The perfusion fixation may be an alternative method for specimen preparation.

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## ABSTRACT

*Background:* The Klingler's method for white matter dissection revolutionized the study of deep cerebral anatomy. Although this technique made white matter dissection more feasible and widely used, it still presents some intrinsic limitations.
*New method:* We evaluated the quality of different methods for specimen preparation based on an intra-carotidal formalin perfusion fixation process. Ten post-mortem human hemispheres were prepared with this method and dissected in a stepwise manner.
*Results:* The homogeneous and rapid fixation of the brain allowed documentation of several fine additional anatomical details. Intra-cortical white matter terminations were described during the first stage of dissection on each specimen. No limitations were encountered during dissection of the major associative bundles. On the contrary, the quality of the fixation of the specimens made it possible to isolate them *en bloc*. One of the most complex and deep bundles (accumbo-frontal fasciculus) was dissected without technical limitations. Deep vascular structures were very well preserved and dissected within the white matter until their sub-millimetric terminations.
*Comparison with existing method:* Short time for preparation, a more homogeneous fixation, no technical limitation for a detailed description of superficial and deep white matter anatomy, the possibility to dissect with a single technique the fibre organization and the white matter vascular architecture are the advantages reported with the perfusion fixation.
*Conclusion:* These results provide encouraging data about the possibility to use a perfusion fixation process, which may help in improving the quality of white matter dissection for research, didactic purposes and surgical training.



## 1. Introduction

The study of cerebral white matter (WM) has always intrigued anatomists of every century since its first description by Galen and the subsequent efforts of Vesalius (Clarke and O'Malley, 1968,1996). Vieussens, Bell, Foville, Panizza, Gratiolet and Meynert among others described many different techniques for specimen preparation and dissection in order to understand the complex WM

*Abbreviations:* WM, white matter; AFF, accumbo-frontal fasciculus; DTT, diffusion tensor tractography; vILF, ventral portion of inferior longitudinal fasciculus; PLI, polarized light imaging; OCT, optical coherence tomography.

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architectural organization (Clarke and O'Malley, 1968,1996; Ture et al., 2000; Agrawal et al., 2011). However, it was only with the contribution of Joseph Klingler that this technique became more feasible and widely used (Ludwig and Klingler, 1956; Ture et al., 2000; Agrawal et al., 2011).

Klingler revolutionized the technique, developing a new method of brain fixation, which consisted of freezing already formalin-fixed brains before dissection (Klingler, 1935). The water crystallization induced by freezing disrupts the structure of the grey matter (which has a high water content), making it possible to peel off the cortex from the brain surface. The freezing process also spreads along the WM fibres, inducing separation between them, which facilitates dissection by progressive peeling of the fibres (Ture et al., 2000; Fernandez-Miranda et al., 2008; Martino et al., 2010).

The ideal goal of fixation, however, is to rapidly and uniformly stabilize cell morphology and tissue architecture, disabling proteolytic enzymes, strengthening the specimen for further processes while protecting them from microbiological contamination and decomposition. Common methods of fixation include perfusion or immersion with chemical fixative agents, freezing and drying (Werner et al., 2000; Unhale et al., 2012).

Klingler improved the quality of the post-mortem brains by merging two of the classical fixation techniques – immersion and freezing – achieving a high level of anatomical detail of the white matter, when the specimens were subsequently dissected.

However several studies on murine and primates brains suggest that, while placing specimens directly in fixative agent works well for small pieces of tissue, larger specimens like the intact brain pose a problem for fixation by immersion because the fixative does not reach all regions of the tissue at the same rate (Koenig et al., 1945; Jonkers et al., 1984; Kasukurthi et al., 2009). Anatomical changes due to hypoxia usually begin before the tissue can be preserved (Zwienenberg et al., 1999). For this reason, if the process of fixation involves a gradual absorption from the superficial layers to the deepest, the deep WM architecture may suffer from this temporal gap of fixative agents as demonstrated by immunohistochemical and autopsy studies in both animals and humans (Rosene and Mesulam, 1978; Beach et al., 1987; Werner et al., 2000).

The preparation process with Klingler's technique takes at least 45–60 days for the brain to be ready for dissection, due to the duration of the formalin fixation process achieved through immersion. According to the classical description (Klingler, 1935; Ludwig and Klingler, 1956; Ture et al., 2000), the concentration of formalin cannot be as high as 10% because of the inhomogeneous penetration through the cortex. A formalin concentration of 5% results in favourable penetration and fixation, but the time needed for the fixation process is increased. Hence a lack of some anatomical details can be potentially associated to the specimen preparation technique. The grey matter, for instance, which is the first layer to be fixed, can easily be removed en bloc, but this results in the destruction of intra-cortical WM terminations. According to the literature, very deep bundles such as the accumbo-frontal fasciculus (AFF) (Rigoard et al., 2011) are better described with modifications of the Klingler technique for specimen preparation. Furthermore WM vasculature, which has been more often studied with transparent specimens and radiological enhancement of thin histological sections, has been difficult to describe during dissection of classical Klingler fixed brains. The vessels are invariably collapsed and too thin and weak to be successfully dissected. This results in a common lack of three-dimensional knowledge of deep vascular structures despite their intrinsic importance.

Many studies on both animals and human brains encourage the use of a perfusion fixation in order to achieve an homogeneous, accurate and fast fixation that can potentially minimize the structural abnormalities due to the long fixation (Koenig et al., 1945; Bondonna et al., 1977; Piechocki, 1986; Adickes et al., 1997; Beach et al., 1987; Eichenbaum et al., 2005; Sharma and Grieve, 2006; Gage et al., 2012). However the majority of these studies evaluated the quality of the specimens fixed with formalin perfusion with neuropathological, histological or immunohistochemical analyses. Only few articles combined the perfusion fixation process of the whole body with white matter dissection of the brain (Lawes et al., 2008; Arnts et al., 2014).

The aim of this paper is to evaluate the use of perfusion fixation process of human brains with a direct intra-carotidal formalin injection for subsequent WM dissections. Our goal is to compare the results provided by this technique in respect to the anatomical accuracy of the standard Klingler technique and whole body perfusion fixation methods reported in literature.

## 2. Methods

### 2.1. Ethical approval

Ten cerebral hemispheres obtained from human cadavers donated to the Department of Medical Cell Biology, Section for Anatomy studies at Uppsala University were enrolled in this study. All individuals donating had given written consent for use of the whole cadaver for biomedical research and education in a testimonial donation letter. The study protocol was filed with the application for ethical vetting of research involving humans to the local ethical review board in Uppsala (Dnr 2014/468).

### 2.2. Perfusion protocol

Each brain was fixed with intra-arterial injection of 12% formalin solution within the first week after death using a perfusion device. After the left or right common carotid artery was exposed and opened at the neck, the pressure transducer was placed within the carotid artery and 2 l of 12% formalin were injected into the intracranial compartment with a perfusion pressure of 200 kPa. The whole duration of the process was approximately 15–20 min. The procedure was considered complete once the ocular bulbs of both sides showed signs of formalin infiltration. After this procedure the brain was considered fixed after 48 h.

### 2.3. Specimen preparation

The brains were carefully extracted and put in 10% formalin for 24 h. The pia mater, arachnoid membrane and vascular structures were then carefully removed under microscopic magnification and the hemispheres were frozen at −15−−20 °C for 6–10 days, then slowly defrosted for 12 h. Before the start of dissection, the superficial anatomy of the sulci and gyri was studied in detail. The specimens were dissected in a stepwise manner, from lateral surface to the medial structures and from the basal surface to the ventricle, with a modified fibre dissection technique in respect to the technique described by Klingler (Latini, 2015). Microscopic metal dissectors and thin wooden spatulas were used in the initial steps of the dissection, to split or partially peel away the brain cortex, preserving the most superficial intra-cortical and subcortical fibres of the lateral and basal brain surfaces. Subcortical, intra-lobar, associative and projection fibres were exposed until the basal ganglia region was reached in each specimen. The vasculature within the WM was (when possible) preserved and dissected until its sub-millimetric subdivision. The dissections were performed under microscopic magnification (up to 4×). Between each dissection session the specimens were placed in 5% formalin.