REVIEW ARTICLE

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Perfusion Fixation: A Vital Process for Neuroanatomical Research

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ABSTRACT

Perfusion fixation is a vital technique that preserves tissues, also preparing them for further techniques in histology, histochemistry, immunohistochemistry and electron microscopy. The need for proper preservation of neural tissues for morphological and immunoreactivity studies led to the discovery of this technique which mostly makes use of the vascular system to allow for uniform tissue preservation. The technique makes use of electric pressure pump or gravitational force for perfusion based on the available equipments, and the type of fixative used is determined by the type of analysis to be done. However, this method is limited when tissues other than the target are required, or when there is a delay in tissue perfusion.

Keywords: *Perfusion, Fixation, Vascular System, Neural tissues*

INTRODUCTION

Perfusion, the act of introducing a fixative through the bloodstream in order to reach internal organs or tissues is a widely used process to preserve different body tissues in life-like form. Perfusion fixation technique not only preserves body tissues in life-like form, but ensures that there is little or no risk of tissue autolysis. This method described as early as 1962 (Palay et al. 1962), removes blood from organs of interest and rapidly and uniformly preserves tissues for further assessment. This technique has been applied across a wide range of mammals and non-human primates, as well as reptiles (Tao-Cheng et al. 2007; Kasukurthi et al. 2009; Manger et al. 2009; Ettrup et al. 2011; Hoops 2015). Its use with these animal species is critical and species specific because the applied physiological pressures are dependent on the animal size and species used (Ikeda et al. 1991). One advantage of direct perfusion fixation through the circulatory system is that the fixative can quickly reach every tissues of the organism using the natural vascular network. So, in order to utilize the circulatory system most effectively, care must be taken to match physiological pressures to the animal size and species (Ikeda et al. 1991).

Perfusion fixation is vital as it preserves tissues, while also preparing them for further techniques in histology, histochemistry, immunohistochemistry and electron microscopy among others. Perfusion fixation technique varies depending on the animal size, target tissue to be fixed and the subsequent processing following fixation. It is also dependent on the facilities available in a given laboratory, where either a pressure pump or gravitational force is applied. It is a better alternative to fixing tissues by immersion in the preservation of the nervous tissues (Morest and Morest 1966; Tao-Cheng et al. 2007; Ettrup et al. 2011). Placing tissues directly in fixative works well

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for small pieces of tissue, but larger specimens like the intact brain pose a problem because the fixative does not reach all regions of the tissue at the same rate (Jonkers et al. 1984; Kasukurthi et al. 2009). Aside the fact that the time taken for the brain to be removed may impact negatively on the tissue viability, often, changes in response to hypoxia begin before the tissue can be preserved (Zwienenberg et al. 1999). Thus, the optimal preservation of large blocks of body tissues ordinarily requires perfusion fixation at the time of euthanasia (Tao-Cheng et al. 2007; Kasukurthi et al. 2009; Manger et al. 2009; Ettrup et al. 2011).

In diagnostic neuropathology, preservation of brain specimens is typically by immersion fixation. Obviously, immersion fixation delays tissue preservation, especially in deeper structures. To parry this, the tissues are cut to expose the deeper structures. However, due to the fragile nature of the unfixed tissues, some of its parts may be damage coupled with the parts already damaged by excessive handling.

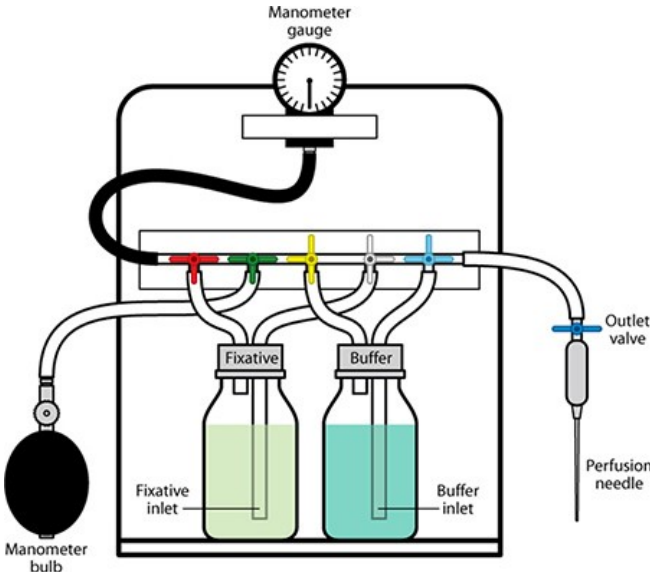

Fig. 1: A perfusion apparatus (Gage et al. 2012)

WHAT DOES PERFUSION FIXATION ENTAIL?

There are two commonly used methods for tissue perfusion, and this depends on how equipped the laboratory is. In well-equipped laboratories, perfusion is by the use of an electric pressure pump whose pressure of perfusion is controlled by a manometer. In less equipped laboratories, gravitational perfusion is carried out. In this instance, perfusion makes use of the height of perfusion fluid tank to determine the pressure. Here, the fixative reservoir is placed such that the top of the fluid level is at a height of approximately 100-120 cm above the level of the heart or artery into which the fixative is to be introduced. The fixative is then introduced into the cardiovascular system (CVS) by gravity drip where fluid is delivered at a pressure of 120-150 mmHg which is approximately equal to systolic blood pressure, and likely to keep the vessels of the central nervous system (CNS) patent.

Tissue perfusion requires some level of skills and experience with the necessary equipments. After animal surgery with the animal under the influence of anaesthesia, the left ventricle of the heart is located and the appropriate pressure is used to avoid tissue damage. Sporadic fluctuations in pressure may lead to closure of small blood vessels in the CNS, which will prevent optimal fixation. On the other hand, too much pressure may cause pale areas due to excessive fluid leakage through the CNS capillary walls.

As there is no reported standard equipment for perfusion technique, each laboratory fabricates its

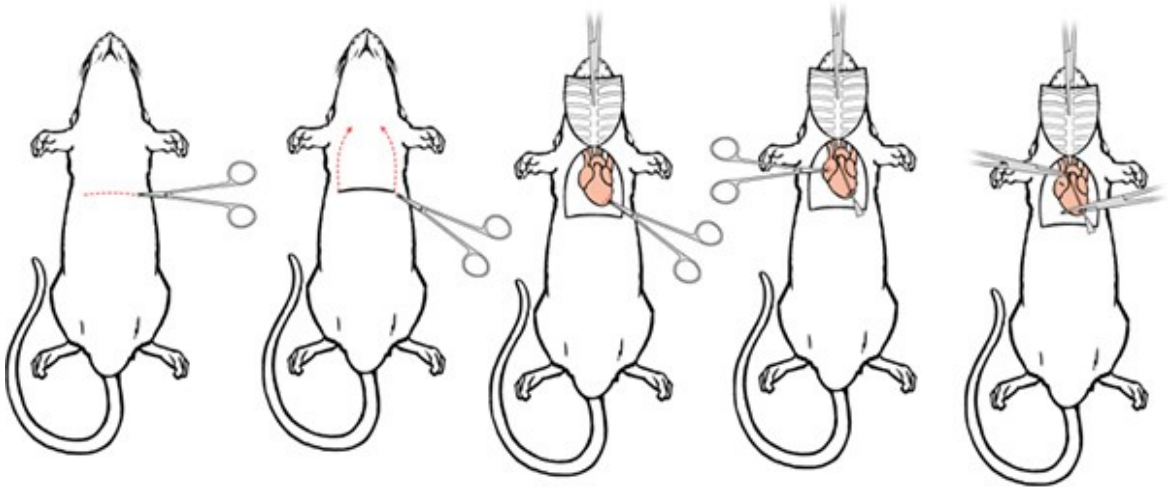

Fig. 2: Thoracotomy to access the heart (Gage et al. 2012)

apparatus to suit its need, with little uniformity to each other, although the functioning principles remain the same. A typical apparatus for perfusion is shown in Figure 1. Fixatives are usually delivered through flexible, clear tubing controlled by two valves system; the first valve sets the intended flow rate of the fixative, and is not changed unless the flow rate requires adjustment. The second valve serves as a stopcock to regulate the flow. Alternatively a three-way stopcock set-up can be used so that each solution has a separate line that can be independently closed (Aminoff and Daroff 2014).

Depending on what the researcher intends to achieve, different perfusion fluids are available for use; these include formaldehyde, paraformaldehyde and glutaraldehyde among others. Formaldehyde (CH_2O), an aldehyde is an important perfusion fluid used as formalin and can also be used as buffered formalin. It penetrates tissues rapidly and reacts slowly with the side-chains of proteins to form reactive hydroxy-methyl groups. It also penetrates nuclear proteins and nucleic acids stabilizing the nucleic acid-protein shell and modifying the nucleotides by reacting with free amino groups. Formaldehyde reacts with some groups in unsaturated lipids particularly if calcium ions are present, but tends to be unreactive with carbohydrates (Eltoum et al. 2001b). It is widely used as a fixative of choice due to its numerous applications as a single or compound fixative. Its use in immunohistochemistry though reported to preserve brain tissues adequately, is limited due to the presence of impurities which can impact on the preserved tissues and further processing techniques (Adickes et al. 1997; Eltoum et al. 2001a).

Paraformaldehyde ($OH(CH_2O)$, a highly polymerised form of formaldehyde is a better alternative as a perfusion fluid for immunohistochemistry. It is mostly used as 4% paraformaldehyde, and is a widely used fixative for perfusion fixation and for further histological and immunochemical technique because of its purity and rapid penetration of tissues (Leong 1994).

Glutaraldehyde ($CHO(CH_2)_3CHO$), another aldehyde though not use routinely, is a good perfusion fixative for electron microscopy (Wisse et al. 2010; Gage et al. 2012). Glutaraldehyde causes more extensive cross-linked in tissues than other formaldehydes, and will also possess some unreacted aldehyde groups that, unless chemically blocked, can cause background staining (Bozzola and Russell 1992). The extensive cross-linking adversely affects immunohistochemical staining but does provide excellent ultrastructural preservation which explains its extensive use as a primary fixative for electron microscopy. While cross-linking reactions of glutaraldehyde are largely irreversible, it penetrates fairly slowly (Bozzola and Russell 1992; Wisse et al. 2010; Gage et al. 2012). The above mentioned aldehydes-base fixatives are being reviewed and do not rule out other fixative types.

PERFUSION ROUTES

Perfusion fixation is usually through the cardiovascular system, but hollow cavities such as the oesophagus, intestines, renal tubules and the ventricular system of the brain have also been used (Lyon 2012). As perfusion fixation makes use of the vascular system, different parts of the vascular system can be explored in rodents. It may be through the descending aorta or vena cava, if the target is the whole animal. However, if a specific system of the body is the target; the aorta, via the left ventricle is used; for example, the central nervous system and pituitary gland, while the descending aorta proximal to its distal bifurcation is used for kidney perfusion, and the portal vein is used for the perfusion of the liver (Wisse et al. 2010; Lyon 2012). Nevertheless, the aorta route has been described as the best possible preservation of the brain for immunohistochemistry and other special histological techniques (Gage et al. 2012). In larger mammals such as the proboscidean, the carotid arteries have been used successfully to perfusion-fix the brain (Manger et al. 2009; Jacobs et al. 2010). Irrespective of the exposure site, the ultimate route for perfusion fixation of neuronal tissues remains the circulatory system.

The CNS may be fixed by whole-body perfusion or by head-only perfusion. In the whole body perfusion technique, the entire blood volume is replaced with fixatives, and therefore the central and peripheral nervous system should be well preserved. Fixative is typically introduced into the left ventricle or base of the aorta and allowed to flow throughout the body. For the head only method, either the thoracic aorta is clamped below the carotid artery origins or the carotid arteries are cannulated bilaterally so that fixative is directed preferentially into the cranial tissues. Only the brain is well fixed in this setting, hence the whole body perfusion is usually preferable (Aminoff and Daroff 2014).

PERFUSION PROTOCOL FOR A RODENT

A good number of laboratories work with rodents, and it is based on this that a rodent is used as example in this review. The rodent is usually anaesthetized with the anaesthesia of choice. A well-anaesthetized animal is indicated by the loss of sensory/reflex response, that is, non-response to tail pinching, or paw pinching.

On complete anaesthesia, thoracotomy is quickly carried out; Using a large, sharp scalpel blade, incisions are made through the skin and muscles of

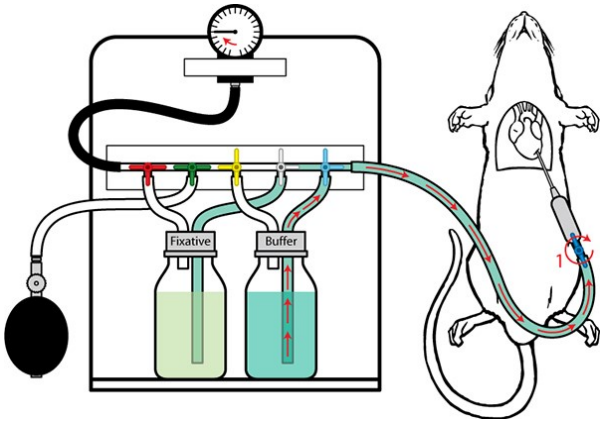

Fig. 3: Perfusion (Gage et al. 2012)

the thorax on each side of the body along the mid-axillary line, extending from the axilla to the inferior margin of the rib cage. A third incision is made across the ventral aspect of the trunk at the level of the xiphisternum. A sharp scissors is used to cut through the abdominal muscles along the margin of the incisions, and haemostats are used to retract the rib cage superiorly and hold it out of the field (Figure 2), (Gage et al. 2012).

A transverse incision through the posterior left aspect of the heart opens into the left ventricle. The heart is then stabilized with forceps and a perfusion cannula inserted through the opening in the left ventricle into the ascending aorta. The cannula is clamped into place with a haemostat across the superior part of the ventricles (Figure 3).

The animal is first perfused with a buffered saline, mostly phosphate buffered (PBS) by turning up the pump or in the case of the gravitational method a drip set valve is essential in controlling the pressure for the saline solution to flow into the aorta. At this point a small incision is made on the right atrium to allow for the perfusion fluid to flow out. As soon as the fluid escaping from the atrium becomes clear, the pump or valve is turned off, and the pump input line from the saline solution is quickly transferred to the fixative and pumping is resumed at a selected rate. As the fixative solution enters the body and the muscles begin to fix, the animal exhibits vigorous muscle contractions and becomes rigid (Gage et al. 2012). This is indicated by the stiffness of the tail and other body parts.

Organs can then be harvested. The brain is removed and post-fixed, that is, placed in a vial of the same fixative containing fluid at least ten times the volume of the brain for the complete fixation period, washed with phosphate buffered saline and stored in phosphate buffered saline with sodium azide and kept at 4 °C. An initial indicator of the success of the perfusion is the clearing of extremities such as the nose, ears, and paws and internal organs such as the thymus gland and liver. Gross inspection of the brain reveals the blood vessel void of blood (white to pale yellow appearance), (Figure 4b). However poor perfusion leaves a reddish hue of blood cells remaining in the brain, and sometimes some soft areas of tissue and shrinkage of soft tissues (Figure 4c). Because red blood cells autofluoresce, they may interfere with cell labelling reactions.

During gravitation perfusion, mice may take 10-20 minutes to perfuse thoroughly, requiring 10-25 ml of saline and 50-100 ml of fixative. Rats may take 10-30 minutes for 50-100 ml of buffer and 400-600 ml of fixative. In the instance of the pressure pump, perfusion pressure should be maintained between 60 and 100 mm Hg, this is achieved by means of a sphygmomanometer, and should last for 5 minutes. Thus, the gravitational technique takes longer, but the results are reproducible and perfusion is thorough. Gravity systems allow consistent pressure and controlled flow rates, providing good perfusion of the major organs. However, excessive pressure may cause artefacts in the brain histology.

Perfusion generally is less economically due to the fact that more fluid is expended compared to the immersion technique with its greatest impediment being the inability to effectively scavenge the waste fumes and fluid (Aminoff and Daroff 2014). Intracardial perfusion is advantageous, but its usefulness is limited if other tissues aside the central nervous system is the target. For instance, instillation of fixative solutions into the airways and in situ fixation preserves the lungs more than transcardial perfusion (Hausmann 2006).

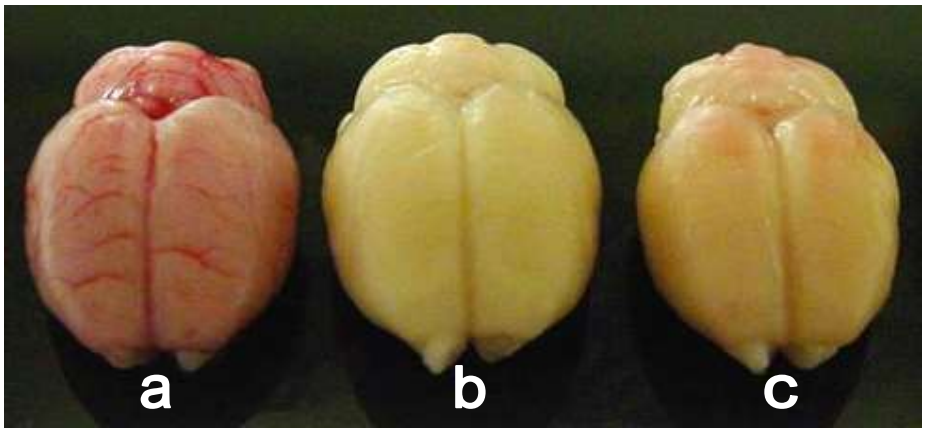

Fig. 4: Rodent brains. a. A non-perfused brain containing blood cells; b. A well-perfused brain devoid of blood cells; c. Reddish hue of blood cells remaining in the brain due to poor perfusion (Scouten 2009).

However, there are several

advantages of perfusion over immersion and/or other technique. Perfusion fixation has been reported to be important for specific staining techniques as it allows for further tissue processing with other techniques which would have been difficult or problematic with any other tissue fixation method. It is reported to preserve morphological details of neuronal processes, dendritic branchlets and spines, and elements of the axonal plexuses. These were shown to be better preserved, and clearly and consistently demonstrated with this technique than in other fixation methods (Morest and Morest 1966). Large, complex spiny neurons were clearly revealed in the proboscideans that were perfused in situ through the carotid artery (Jacobs et al. 2010).

Even with fixatives other than the aldehydes, perfusion remains vital for neuronal demonstration. Perfusion fixation with osmium tetraoxide-dichromate have been reported to aid rapid and more uniform and extensive Golgi impregnations of neurons in the brains of opossums, rats, rabbits, chinchillas, and cats, which yielded Golgi impregnations than in immersion-fixed and formalin-perfused preparations (Morest and Morest 1966; Reith et al. 1984).

Though perfusion fixation is indispensable for brain tissue preservation, structural changes may still occur in the brain when there is a delay in perfusion, and this has been reported to provide a means to assess perfusion fixation quality in experimental structural studies of brain (Tao-Cheng et al. 2007). They reported that delay in perfusion fixation resulted in a condition that mimics ischemic stress in the normal brain. These structural changes may be used as a subtle diagnosis on the changes that occur in intact brain following metabolic stress, when there is inadequate supply of blood.

CONCLUSION

Perfusion fixation is very necessary for preservation of tissues, especially the nervous tissue and for further processing making use of the cardiovascular system. This method is limited when tissues other than the nervous tissues are the target, or when there is a delay in tissue perfusion.

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