Tissue sectioning for epifluorescence microscopy

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Specimens labelled with multiple fluorophores illuminating a variety of cellular targets, facilitated by developments in probes, data acquisition and analysis technologies, have extended the capabilities of anatomical investigations. However, the field has largely neglected development and consensus in optimal techniques of specimen preparation.

The optical properties of the specimen largely determine study outcomes in epifluorescence microscopy. Thus, we discuss tissue preparation protocols and their application in multiple anatomical investigations. The processing of tissues optimised for multiple studies are detailed. The conservation of cyto-architecture and antigenicity, reduced tissue autofluorescence, increased permeabilisation and applications for thick tissue sections are examined. The application of multiple labelling immunofluorescence, fluorescent in situ hybridisation and fluorescent histochemistry, in qualitative and quantitative analysis are considered.

Keywords Anatomy; Frozen sections; Fluorescence histochemistry; Immunofluorescence; in situ hybridisation; Microscopy; Morphometry; Paraffin; Polyethylene glycol; Stereology; Tissue sectioning.

1. Anatomical techniques: strengths and limitations

Anatomical studies of cellular and subcellular structures are widely used in all arms of biomedical research. It is recognised that accurate visualisation of microscopic structures is limited by the physical properties of light; the interaction of light with the specimen and the capabilities of the instrumentation. Notably, recent advances in illumination technologies, light emitting probes, detection instrumentation and computerised data analysis has led to a revolution in anatomical techniques [1,2].

The visualisation of cellular structures within live animals is used in relatively small and specialised fields. These applications are limited by the impact of light-scattering tissue surrounding the object of interest restricting light penetration and access by optics. Frequently, tissue interference is minimised by sectioning living tissue, allowing the study of physiological processes. This rapidly advancing area of live cell microscopy is also limited when analysing tissue and cellular structure at high resolution due to specimen instability. Therefore the majority of anatomical studies use non-living tissue for imaging, with tissue stabilisation through fixation and the constraint of interfering tissue is minimised through fine dissection for whole-mount preparations or tissue sectioning to isolate specific regions of interest [3].

Multiple sectioning protocols are available for processing fixed tissue and their selection is largely determined by the demands of the analytical protocols being applied. For example, electron microscopy requires electron-dense probes which demand ultrathin sections in the submicron ranges, whereas tissue sections with thicknesses ranging from 1-20 µm are often used in histochemical studies.

The advent of fluorescence probes has facilitated the visualisation of structures embedded within a translucent specimen. Furthermore, multiple probes may be used to target several structures of interest and their associations, within a given specimen. The ability to image within a tissue, has in theory, circumvented the need to physically isolate fine regions of interest, facilitating the analysis of cellular and subcellular associations. Thus, there is a recognised need and growing interest for high resolution imaging within thick specimens [4], with a need for maintaining good tissue ultrastructure.

Several techniques have been developed to optimise tissue preparation and sectioning protocols which facilitates imaging within thick specimens [4-7]. The increased specimen path length when imaging within thick specimens demands minimal tissue autofluorescence and light scatter to facilitate high resolution imaging. Furthermore, advances in several fluorescent labelling techniques including immunofluorescence, fluorescence in situ hybridisation, fluorescence histochemistry and fluorescent reporters have driven the development of tissue preparation protocols for multiplexing epifluorescence imaging. The utility of these techniques are presented below in the context of multiple anatomical investigations in the mammalian brain and spinal cord.
2. Investigating endogenous reporters and vital dyes in unfixed tissue specimens

In reviewing tissue sectioning for epifluorescence microscopy, the study of endogenous reporters and vital dyes in living tissue, a growing field of interest, requires mention. Investigations analysing cellular physiology with endogenous reporters and vital dyes frequently use unfixed sectioned tissue. Briefly, following tissue harvest from live animals further fine tissue dissection or a “tissue chopper” may be used to reduce the specimen thickness for effective imaging. Gelatine or agarose embedment matrices and vibratome or sledge microtome sectioning are also used to generate live tissue sections. Whilst live cell imaging may provide a unique window into cellular physiology, these techniques have limited capability in high resolution imaging. These rapidly advancing areas of microscopic investigations are outside the scope of this article and have received previous comprehensive review [8]. Similarly, the use of unfixed tissue sections for investigating the localisation of endogenous reporter expression will not be discussed in this article.

3. Investigating fixed tissue specimens

Biological tissues cause extensive light scattering which has constrained high resolution fluorescence microscopy to the superficial layers of a specimen and the imaging of thin tissue sections. As many studies do not require three dimensional data at a cellular level, imaging from thin sections and cell monolayers are adequate. Where imaging within thicker specimens is needed, several techniques including laser scanning confocal microscopy, structured wide-field microscopy and deconvolution techniques have circumvented these limitations to a greater extent. Notably, precise within thicker specimens is needed, several techniques including laser scanning confocal microscopy, structured wide-field epifluorescence microscopy [5-7]. For fluorescence imaging in tissue sections using fluorescence probes, fixation, optical clearing, sectioning and mounting of the specimen, requires careful attention to detail. We discuss here techniques developed for high resolution imaging of thick brain sections using multiple fluorophores.

3.1 Tissue harvesting and fixation

In preparing laboratory animal tissue for fluorescence microscopy, transcardiac perfusion with 0.1M phosphate buffered saline (PBS) is routinely used to clear autofluorescent red blood cells from the specimen. When harvesting tissues from rats or mice we routinely use 0.5% sodium nitrite in 0.1M PBS as a vasodilator to facilitate removal of red blood cells and facilitate perfusion. Perfusion rates which minimise rupture of fine blood vessels (4mL/min and 1mL/min for adult rats and mice, respectively) are used to minimise entrapment of red blood cells within tissues and minimise damage to tissue structure. The specimen is then perfused with fixative. Transcardiac perfusion provides greater penetration by the fixative and even fixation of the tissue in comparison to immersionfixed tissue. We routinely perfusion-fix brain tissue, followed by 2-24 hours immersion-fixation in vacuo, at room temperature.

The stabilisation of a specimen may be achieved using several types of fixatives. Fixatives range from simple alcohols such as methanol which primarily increases hydrogen bonding within the tissue, to aldehyde fixatives which covalently cross link amine groups within proteins through the formation of methylene bridges [9]. Fixation can markedly alter the structure of protein causing a loss of antigenicity or an increase in tissue autofluorescence [10, 11]. We routinely use freshly made 4% formaldehyde (from paraformaldehyde; PFA) in 0.1M phosphate buffer saline (pH 7.2). PFA based fixatives are widely used in immunofluorescence studies as most antigens are minimally influenced by formaldehyde fixation [12] and in the case of some antigens, the antibodies may be raised against formaldehyde treated proteins. Together with good tissue penetration it is considered a robust fixative [13, 14]. However, care in the preparation and storage of formaldehyde based fixatives is needed, as polymerisation may result in increased tissue autofluorescence [15].

3.2 Optical clearing

High resolution imaging of mammalian tissue is constrained by its inherent light scattering properties. This is compounded in imaging tissues from the central nervous system due to its high myelin content and resultant increased opacity [5]. These difficulties may be reduced by the application of appropriate tissue clearing protocols and mounting media. Robust tissue clearing, which involves the treatment of tissues with solvents that dissolve lipids, assists in the removal of optically dense material and permeabilises the tissues. This allows the mounting media to permeate the specimen, creating an even optical density and consistent light path through the specimen and mounting medium [5, 7, 16]. Optimising these protocols is especially pertinent when capturing high resolution data from thick specimens of the central nervous system, as required in our laboratory.

Following fixation (Section 3.1), we routinely remove the fixative from tissues by extended washes in PBS containing 0.1% Triton X-100, followed by further tissue clearing by washing in a 100% dimethyl sulfoxide (DMSO) solution for 10-60 minutes as determined by the specimen thickness [6, 17, 18]. A similar protocol using Dent solution containing 30% DMSO, demonstrated reduced optical density and thereby better imaging from thick tissue sections [5]. This seemingly harsh tissue clearing/permeabilisation step has several benefits. Whilst the detergent, Triton X-100 effectively dissolves lipids and assists in the removal of optically opaque materials, DMSO interacts with the lipid...
membrane, creating water pore formation and displacing water [19, 20]. This alteration to the cell membrane structure facilitates the removal of water from the extra and intracellular space and the infiltration/impregnation of the tissue with the mounting medium. We further facilitate these processes (due to the high viscosity of the solutions) using vacuum and bench top rockers (at approximately 1 hertz) to lower the surface tension of the liquids and prevent the formation of diffusion gradients respectively.

This protocol is suitable in studies targeting large molecules which are not readily washed out of tissues, with appropriate controls required when investigating cell surface markers and unbound low molecular weight targets. All antigens, which are detectable in specimens cleared using other permeabilisation/clearing protocols such as saponin, Triton X-100 alone or glycerol washes, were detected using the protocols as discussed [6, 17]. Further, following these fixation, permeabilisation/optical clearing protocols, antigen retrieval is not required.

3.3  Sectioning

Biological tissue usually requires the support of a matrix to facilitate sectioning. In general, the thinner tissue sections require embedment with dense matrices (resins, paraffin wax, freezing media), whereas thick tissue sections may be sectioned held within a less dense substrate (e.g. polyethylene glycol (PEG), agarose or gelatin) or even in the absence of a surrounding matrix (directly glued onto the chuck or with a “tissue chopper”).

The majority of tissue embedment protocols require the removal of water from tissues and its replacement with the embedment medium that hardens allowing sectioning. Water, the primary constituent of biological tissue is removed in the process of dehydration, where tissues are transferred through washes of ethanol of progressively increasing concentration. Following dehydration the ethanol is removed using a clearing agent such as xylene or DMSO.

3.3.1 Thin sections and cell monolayers: Small specimen path length

Thin sectioning is often used to reduce the specimen to a single layer of cells of interest thus allowing investigation of cellular and subcellular structures. This may be in the range of 1 to 10 µm for epifluorescence microscopy. To achieve this, tissues are stabilised by impregnation with acrylic resins whose polymerisation is catalysed by heat, ultraviolet light, or through chemical induction, e.g. paraffin wax. The freezing of liquids or water-based embedding material such as glycol, TBS, or OCT (Optimal Cutting Temperature media) forms hardened blocks, providing tissue rigidity. Tissue freezing is frequently preceded by fixation.

Resin embedded tissue offers the advantage of superior cell morphology conservation over frozen tissue. Paraffin embedded tissue sectioning is therefore widely used, particularly in clinical pathology [21]. This method can be used in histochemical, in situ hybridisation and some immunohistochemical applications. However the widespread disruption of antigens due to the wax impregnation protocol [22, 23] and the inherent high tissue autofluorescence further restrict its application in immunofluorescence microscopy. Notably, some antigens may be rescued through varied antigen retrieval protocols [24, 25]. Whilst antigen retrieval may unmask some antigens, they may damage others. More recent optimisation of antigen retrieval protocols provided greater promise for immunofluorescence and immunohistochemical studies [26, 27]. Despite these advances, we have found the availability of antigens remain restricted and show greater variability in paraffin embedded tissues in comparison to sections generated from frozen and PEG embedded specimens. Sections generated using our revised PEG embedment and OCT embedment protocols (Section 3.3.2) provide optimal antigenicity and tissue ultrastructure due to their low autofluorescence and high permeability and are thus used to generate thick free floating tissue sections as will be discussed in the subsequent section.

Whilst antigens are well conserved and multiple labelling immunofluorescence in standard frozen tissue sections allows resolution of multiple cellular targets due to low tissue autofluorescence, cellular and sub-cellular structures are often disrupted [28, 29]. This is largely due to cell lysis from water crystallisation, when imparting rigidity to soft tissue by freezing. Therefore, frozen sectioning has commonly been applied where detection of antigens using immunofluorescence for cross correlation of multiple target molecules has taken priority over cellular structure. We have optimised protocols to conserve cellular ultrastructure in frozen tissue sections following application of the optical clearing protocols, as detailed previously. This protocol is used to generate thick adherent tissue sections and will also be discussed in the subsequent section.

Paraffin embedded and standard frozen tissue sectioning is applied extensively in biomedical research using well established protocols. These techniques require modification or replacement by other techniques for applications with particular needs such as morphometric analysis. In order to maximise outcomes in studies requiring multiple analytical approaches there is a need for tissue sectioning techniques, which facilitates multiple parallel applications.

3.3.2 Thick sections and cell aggregates: Large specimen path length

Following tissue harvesting, fixation and optical clearing protocols as discussed previously (Sections 3.1 and 3.2), we generate specimens with optimal structure, permeability, and optical clarity, together with low tissue autofluorescence. We extend this to two tissue sectioning strategies to obtain free floating sections optimised for cellular ultrastructure
and even penetration of fluorescence probes (PEG embedment), or for the generation of adherent tissue sections with good cellular structure for cell quantification (OCT embedment).

PEG embedment

We refined the protocols for PEG impregnation [6, 18]. Briefly, the brains are dissected clear and post-fixed under vacuum. The specimens are then dehydrated through an ethanol series before being placed in 100% DMSO for 45-60 minutes rinsed and immersed in fresh 100% ethanol for a further 60 minutes. They are placed in PEG 400 (Molecular Weight 400 solution) overnight, at room temperature, in vacuo and subsequently placed in a pre-melted solution of PEG 1000 which is placed in a vacuum oven at 48°C for one hour. The specimens are then transferred into a pre-melted solution of PEG 1450/1000 mixed at ratios ranging from 3:1 to 7:1 (depending on the temperature and humidity in the lab) and placed in a vacuum oven at 48°C until they sink (approximately 1 hr). The specimens are removed from the impregnation solution and oriented in a cryo-mold containing fresh PEG 1450/1000 mix at the same ratio as the impregnation solution. The specimens are placed in a sealed container with silica gel and set at 4°C ensuring the absence of air bubbles. The block is removed from the cryo-mold and attached to a microtome specimen holder or embedding cassette base, using the PEG 1450/1000 mix.

The blocks are cut at room temperature on a rotary microtome at thicknesses ranging from 10 to 60 μm. The “free floating” sections, are placed ten sections per well in 0.1M PBS containing 0.05% sodium azide (PBS azide). They are sealed and stored at 4°C for up to two years. Uncut blocks may be stored for over three years prior to sectioning.

OCT embedment

The brains are dissected clear and post-fixed under vacuum. The tissues are then washed twice with 0.1M PBS and permeabilised in DMSO for one hour followed by two washes in 0.1M PBS for 30 minutes. The tissue was placed in 30% sucrose in PBS azide and placed in the fridge overnight before being placed in a series of OCT solutions of increasing concentration (20%, 30%, 50% and 70% in 30% sucrose in PBS with azide) for one hour each. The brains are then mounted in 100% OCT and stored at -80°C until sectioning. The tissue is sectioned at 10 to 60 μm using a cryostat. Sections are kept as free floating sections in PBS azide or as adherent sections on coated slides at 4°C until processed. All washes and incubations are done in vacuo at room temperature.

3.4 Mounting media

The optical properties of thick tissue sections, improved by optical clearing and tissue permeabilisation can be further improved by permeation of the tissue by mounting media which maximise light transmission [7].

As discussed under section 3.1.2, the protocols applied for optical clearing which involve treatment of tissues with solvents which dissolve lipids and results in water pore formation maximises the permeabilisation of the tissue. The immersion and penetration of the tissue sections in an immersion media with a refractive index (n) matched to that of the immersion oil circumvents depth and sample induced aberrations [30]. This is especially critical when we image using high numerical aperture (NA 1.40) oil immersion lenses (63X objective), from thick tissue samples where the focal plane is extended deep into the tissue (long path length) for the generation of three dimensional data [31].

Furthermore, the protocols that we apply for the preparation of thick sections of brain tissue, promotes the penetration of the tissue with a high numerical density mounting medium (n 1.457; Vectashield DAPI [4',6 diamidino-2-phenylindole], Vector Labs), circumventing a mismatch of the refractive index of the specimen and lens immersion media (n 1.518) [32]. This further facilitates high resolution imaging [7].

3.5 Image capture

Laser-scanning confocal microscopy using several platforms is used for analysis; however wide-field microscopy with structured illumination is routinely used in our laboratory for data capture where large data sets from thick tissue sections, using multiple fluorescence probes are required.

Briefly, the specimens are visualised on an Axio Imager Z1 epifluorescence microscope with ApoTome, using 5X Plan-Neofluar and 20X Plan-Apochromatic objectives (NA 0.15 and 0.75, respectively) and an oil immersion 63X Plan-Apochromatic objective (NA 1.40; Carl Zeiss, Germany). An automated stage, AxioCam Mrm camera using AxioVision software run on a dedicated computer facilitates rapid automated data acquisition. Three dimensional data are manipulated and analysed using AxioVision (Carl Zeiss, Germany) and Imaris software (Bitplane, Switzerland). Figures are compiled in Adobe Photoshop 11.1 and Adobe Illustrator 14 (Adobe Systems Incorporated).

The tissue preparation and sectioning protocols as detailed are widely applicable to imaging systems optimised for three dimensional image capture.

3.6 Tissue sections in multiple anatomical investigations

We apply the thick tissue sections generated using the protocols as discussed above in multiple anatomical investigations in the brain and spinal cord of rats, mice, guinea-pigs, primates, and humans.
The tissue sectioning protocol using PEG impregnation maximises the conservation of cellular structure allowing fine cellular and subcellular elements to be readily resolved. Our data on fluorescence probing of PEG sectioned brain corroborates previous findings on the conservation of ultrastructure. The ability to use multiple markers on the same specimen together with the conservation of cellular structure allows detailed resolution of structural associations between target antigens. As in Figure 1, the PEG tissue sectioning resulted in low tissue shrinkage and minimal autofluorescence and is therefore conducive to quantitative assessment of cellular structures labelled with multiple fluorescence probes in thick tissue sections. Such specimens are optimal for three dimensional morphometric and stereological analysis, currently used in our laboratory for accurate quantification of cells targeted using fluorescence probes [33].

Both sectioning protocols allow the combination of multiple fluorescence probes such as DNA (e.g. BrDU and EdU) and oligonucleotide (e.g. mouse microsatellite) probes together with multiple labelling immunofluorescence for a range of antigens including structural proteins, neurotransmitters, enzymes and membrane associated proteins in tissues that show good structural integrity. As Figure 2 shows, this facilitates the analysis of cellular and sub-cellular associations. Data previously reported by us using this technique has allowed the tracking and accurate quantification of multiple cellular phenotypes within xenografts [6, 18] and in animal models of neurodegeneration [17].
**Fig. 2** Multiple labelling immunofluorescence within the olfactory epithelium showing the stratified distribution of the mature (green) and immature (red) olfactory receptor neurons, within the olfactory mucosa. Their processes combine to form nerve fibre bundles (red and green colocalising to orange) that traverse the lamina propria, to directly innervate the brain. Bacterial infection of the brain via the olfactory pathway is under investigation using high resolution imaging [34]. Scale = 50µm.

An emphasis on appropriate tissue fixation, optical clearing/permeabilisation and section mounting markedly facilitates microscopic analysis of biological material. Methods facilitating the application of multiple analytical protocols such as, detailed structural analysis combined with cell counts using immunofluorescence and *in situ* hybridisation targeting specific DNA sequences are especially useful. Thus technical breakthroughs in microscopy, such as dramatically improved resolution, automated image capture and imaging of three dimensional objects, are driving the resurgence of light microscopy and may be supported by optimal specimen preparation.

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**References**


