A versatile correlative light and electron microscopy protocol for human brain and other biological models

Notash Shafiei*[1,2] Daniel Stähli*[1,2], Domenic Burger[1,2], Marta Di Fabrizio[1,2], Lukas van den Heuvel[1,2], Jean Daraspe[3], Carolin Böing[4], Sarah H Shahmoradian[5], Wilma DJ van de Berg[6,7] Christel Genoud[3], Henning Stahlberg[1,2], Amanda J Lewis[1,2#].

*these authors contributed equally

Author Affiliations

1. Laboratory of Biological Electron Microscopy, Institute of Physics, School of Basic Sciences, EPFL
2. Department of Fundamental Microbiology, Faculty of Biology and Medicine, University of Lausanne, 1015, Lausanne, Switzerland.
3. Electron Microscopy Facility, Biophore, University of Lausanne, 1015 Lausanne, Switzerland.
4. C-CINA, Biozentrum, University of Basel, Basel, 4058, Switzerland.
5. Brain institute and Cancer Center, UT Southwestern Medical Center, 75235-8823 Dallas, Texas, United States
6. Department of Anatomy and Neurosciences, section Clinical Neuroanatomy and Biobanking, Amsterdam Neuroscience, Amsterdam University Medical Centre, Vrije University Amsterdam, 1081 HZ, Amsterdam, The Netherlands.
7. Amsterdam Neuroscience, program Neurodegeneration, Amsterdam University Medical Centre, Vrije University Amsterdam, 1081 HZ, Amsterdam, The Netherlands.

 # Correspondence to: Amanda J Lewis
Full address: LBEM IPHYS SB EPFL
Cubotron (UNIL),
Rte de la Sorge,
CH-1015 Lausanne,
Switzerland
E-mail: amanda.lewis@epfl.ch

Abstract

Correlative light and electron microscopy (CLEM) combines light microscopy (LM) for target identification using genetic labels, dyes, antibodies, and morphological features with electron microscopy (EM) for high-resolution subcellular structure analysis. We describe an optimized room-temperature CLEM protocol for ultrastructural investigation of post-mortem human brain tissues, which is adaptable to cell lines and animal tissues. This versatile 8-day protocol encompasses sample fixation for optimal ultrastructural preservation, immunofluorescence staining, imaging, and multi-modal image correlation, and is executable within standard EM laboratories. Serving as a critical tool for characterizing human tissue and disease models, room-temperature CLEM facilitates the identification and quantification of subcellular morphological features across brain regions and can act as a preliminary step to assess sample suitability for cryo-EM studies.

Key points

- This protocol for correlative light and electron microscopy (CLEM) is optimized for analyzing post-mortem human brain tissues. It focuses on maintaining the integrity of ultrastructural features, thereby minimizing artifacts and structural alterations.
- Examining post-mortem human brain tissues and disease models at the ultrastructural level is crucial for elucidating the mechanisms underlying neurodegenerative disorders, providing essential data for advancing our understanding of disease pathogenesis.

Introduction

Room temperature electron microscopy (EM) is a powerful tool to investigate biological samples label-free at high resolution down to the nanometer scale, which allows the visualization of cytoskeletal features, cell organelles, vesicular transport, and pathological features such as amyloid fibrils, etc. collectively called “ultrastructure”. However, EM is limited by its field of view. Much larger areas and volumes can be investigated using light microscopy (LM) although conventional LM is limited by its resolution, roughly at 250 nm due to the diffraction limit of light. Additionally, LM predominantly utilizes dyes or fluorescent labeling to selectively illuminate cellular components, constraining the analysis to the spectral capabilities of the microscope, which typically accommodates only 4 to 5 distinct fluorescent

channels simultaneously. With the advent of super-resolution techniques, it has become possible to surpass the diffraction limit traditionally imposed by light microscopy. Techniques such as stimulated emission depletion (STED) microscopy have achieved resolutions as fine as 60 nm laterally[1,2], while single-molecule localization techniques like photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM) and expansion microscopy have reached resolutions of approximately 20 nm[3,4]. However, the resolution offered by EM remains unmatched, allowing for visualization of ultrastructural features at a finer scale, which are not accessible with light microscopy techniques. The integration of LM and EM in correlative light and electron microscopy (CLEM) enables the scanning of large fields of view and targeted investigation of regions of interest (ROIs). Initially identified by LM, these ROIs are subsequently examined at ultrastructural levels using EM. This approach is particularly beneficial for studying rare targets or when specific biochemical characterizations using markers are required, offering significant advantages over using EM alone. Established CLEM methodologies, dating back to the 1960s, have facilitated notable studies such as the development of type 5 adenovirus particles in HeLa and HEp2 cell lines[5,6], and the investigation of synaptic connections in the murine brain[7,8]. In neurodegenerative disease research, CLEM has been crucial for directly investigating neuropathological inclusions such as Lewy bodies in Parkinson's disease[9], alpha-synuclein inclusions in multiple system atrophy[10], TDP-43 inclusions in frontotemporal dementia[11] and tau tangles in Alzheimer's disease and corticobasal degeneration[12].

Combining LM and EM requires meticulous methodology and specific technical expertise. Successful CLEM depends on carefully balancing the preservation of ultrastructure, which is prone to quick deterioration, with effective LM staining that highlights specific targets. Accurately aligning and correlating LM and EM images is crucial for producing complementary data, a process that often relies on advanced software to correct image distortions and scale differences. Advancements have significantly enhanced the capability of CLEM. These include the development of high-affinity antibodies for specific epitopes and posttranslational modifications, which improve specificity and sensitivity. Improved preservation techniques have also extended tissue viability for more detailed analysis. Enhancements in labeling strategies[13], super-resolution fluorescence microscopes[14], faster and higher-resolution electron detectors, and sophisticated image analysis algorithms[15] enable more detailed exploration of complex biological processes and disease mechanisms at the nanometer

scale. These technological improvements are vital for advancing our understanding of biological structures and functions.

Our presented protocol is straightforward and adaptable, designed by a multidisciplinary team with deep expertise in advanced microscopic imaging for studying the post-mortem human brain. It has been effectively used to analyze protein aggregates in various neurodegenerative diseases such as Parkinson's Disease[9], multiple system atrophy[10] and Frontotemporal Lobar Dementia[11]. Additionally, the protocol has been applied to cell culture samples[11,16], post-mortem human skin biopsies and mouse brain tissue (unpublished data, Supplementary Figure 1). It requires minimal specialized equipment, allowing anyone with access to a light microscope, an ultramicrotome and an electron microscope to perform this CLEM pipeline independently.

Development of the protocol

We have successfully utilized CLEM to examine the ultrastructure of Lewy bodies in the post-mortem human brain[9]. Working with post-mortem human brain tissue presents challenges, notably the lack of genetically encoded molecular labels and the risk of ultrastructural damage during permeabilization for immunolabeling[17]. To address these issues, we implemented a post-embedding CLEM approach (Figure 1). This method involved collecting human brain tissue through a rapid autopsy collection, ensuring a post-mortem delay of less than six hours. At autopsy, small blocks of brain tissue (±5 mm^3 volume) were dissected from anatomically defined regions by a neuropathologist or -anatomist and subsequently chemically fixed for electron microscopy to maximize the ultrastructural preservation of the tissue. The blocks were postfixed and sectioned using a vibratome (20-100 micron thick sections), heavy metal stained and flat embedded in resin. Small pieces (~1 × 1 mm) were removed using a razor blade and mounted on resin blocks for serial sectioning. The serial sections were alternately collected on glass slides and EM grids all the way through the tissue. The glass slides were processed for immunohistochemistry with ROIs localized by positive antibody staining detected by LM. EM grids immediately adjacent to the immunopositive light microscopy slides were imaged by transmission electron microscopy (TEM) at low magnification to obtain a map. The resulting LM and EM images were overlaid to define the specific location of the ROIs in the TEM images and direct the collection of subsequent higher resolution TEM images.

Using post-embedding CLEM, we discovered the membranous nature of Lewy bodies in the human brain, a characteristic previously overlooked. However, since our CLEM analysis involved randomly cutting pieces from embedded brain slices without prior knowledge of the pathology's abundance or distribution, many blocks lacked pathological features. This resulted in significant time and resources being spent on the labor-intensive steps of ultramicrotomy and immunohistochemistry (IHC).

We then enhanced our protocol by introducing immunolabeling of vibratome sections, followed by fluorescent-based microscopy and toluidine blue staining for light microscopy[10]. This integration of fluorescence microscopy enables efficient targeting of tissue areas with abundant pathology and allows for detailed fluorescent characterization of specific subcellular ROIs in 3D. Toluidine blue staining aids in effectively correlating signals from fluorescence, light microscopy, and electron microscopy[18]. Additionally, we replaced imprecise manual tissue dissection with laser microdissection, which enables precise excision of ROIs based on the fluorescence signals. These improvements have significantly enhanced our CLEM pipeline, providing the capability to accurately target and analyze regions with sub-cellular precision in the human brain.

Applications of the method

The CLEM protocol presented here has mainly been used for the investigation of different neuropathological aggregates in the post-mortem brain of patients suffering from neurodegenerative diseases. We are applying it also to human skin biopsies, mouse tissues and on cell monolayers. Therefore, we are confident that this protocol can be adapted for a wide range of potential applications in biology: from cell cultures to human tissue. The combination of light and electron microscopy takes advantage of the strengths of both techniques to target a desired biomolecule for high-resolution imaging. The only requirements are functioning fluorescent markers that work on tissue chemically fixed for EM, and/or a target that can be resolved using LM. This protocol is designed to link biological features or targets of interest, characterized using dyes and antibodies, to the sample’s ultrastructure. Here we explain the fundamental protocol step by step on post-mortem human brain tissue that can be used in any EM laboratory. While we use our protocol mostly to correlate LM images with TEM images from serial sectioning, it can be also coupled to correlative volume EM approaches such as serial block face scanning electron microscopy (SBF-SEM) or focused ion beam scanning

electron microscopy (FIB-SEM)[19]. This protocol can also be adapted to collect sections on supports for array tomography (AT) on a scanning electron microscope if the high resolution of TEM is not required[20–22]. It is also possible to use our pipeline to verify the presence of targets of interest at large scale before investing in cryo-EM.

Comparison with other methods

CLEM originated in the 1960s and has been used sporadically for ultrastructural studies of the post-mortem human brain[6,9–11,23–25]. Since its inception, significant advancements have occurred in EM, LM, fluorescent markers—especially antibodies—and tissue preservation techniques[17,26]. Historically, the difficulty of locating ROIs in traditional preparations meant that many studies relied on brain samples prepared for classical histology, involving fixation in formalin, dehydration with methanol/ethanol, and embedding in soft resin, followed by re-embedding in harder resin. This approach often led to the substantial loss of cellular lipids and damage to organelles, such as mitochondrial cristae[17]. With CLEM, however, targeted identification and preparation of ROIs have become feasible, enabling the use of optimized protocols that preserve ultrastructure and improve staining for EM[27–29]. This has facilitated more precise investigations of cellular components under EM.

Several notable CLEM protocols have been developed, each tailored to specific sample types and incorporating unique technical methods. For example, Kopek et al. have developed protocols that integrate various super-resolution fluorescence imaging techniques with EM, primarily for cell culture samples[30]. Bohumil Maco and colleagues have combined LM with FIB-SEM to study synapses in the mouse brain[31], offering detailed volume data despite imaging smaller x-y planes compared to our TEM approach. They utilize a two-photon laser microscope and a method known as near-infrared branding to place fiducials around their ROIs. Other innovative CLEM protocols include the use of dark field microscopy combined with SEM to track endocytosis and the intracellular distribution of spherical nucleic acids[32]. Additionally, fluorescence recovery after photobleaching (FRAP) microscopy paired with EM has been used to examine labeled vesicles in synapses of cultured hippocampal neurons[33]. In neuroscience, volume EM techniques are frequently employed alongside CLEM, leading to numerous volume CLEM protocols, generally based on SEM techniques. Array tomography (AT) is used to enhance labeling on sections[21,22] or to automatically generate large volumes of neuropils at EM resolution[34].

Our protocol employs standard EM and LM techniques and equipment and, while primarily focused on the human brain, is versatile enough to be adapted for studying other human and animal tissues, as well as cell monolayers. We provide detailed guidance on how to preserve tissue ultrastructure and facilitate the correlation between LM and EM images.

Additionally, we acknowledge the development of several recent cryo-CLEM protocols utilizing vitrified samples for *in-situ* structural studies[35–37]. However, cryo-CLEM techniques are currently lower throughput and require specialized equipment (e.g. cryo FIB-SEM and cryo fluorescent microscopes) as well as extensive technical training (e.g. cryo-electron microscopy of vitreous sections (CEMOVIS). Compared to these advanced methods, our protocol offers a quicker, more cost-effective alternative. It requires less specialized equipment and provides a practical initial step for assessing the feasibility of a project for cryo-CLEM.

Experimental design

This protocol describes how to investigate the ultrastructure of post-mortem human brain tissue using CLEM. The procedure is illustrated in Figure 1. We also included details where the protocol would differ significantly for the investigation of mouse tissue and cell cultures. We used software plugins, developed custom-scripts and used specific instrumentation to maximize precision and automation where possible. However, these automation steps are not essential to the protocol and can be done manually. We additionally included the details for this.

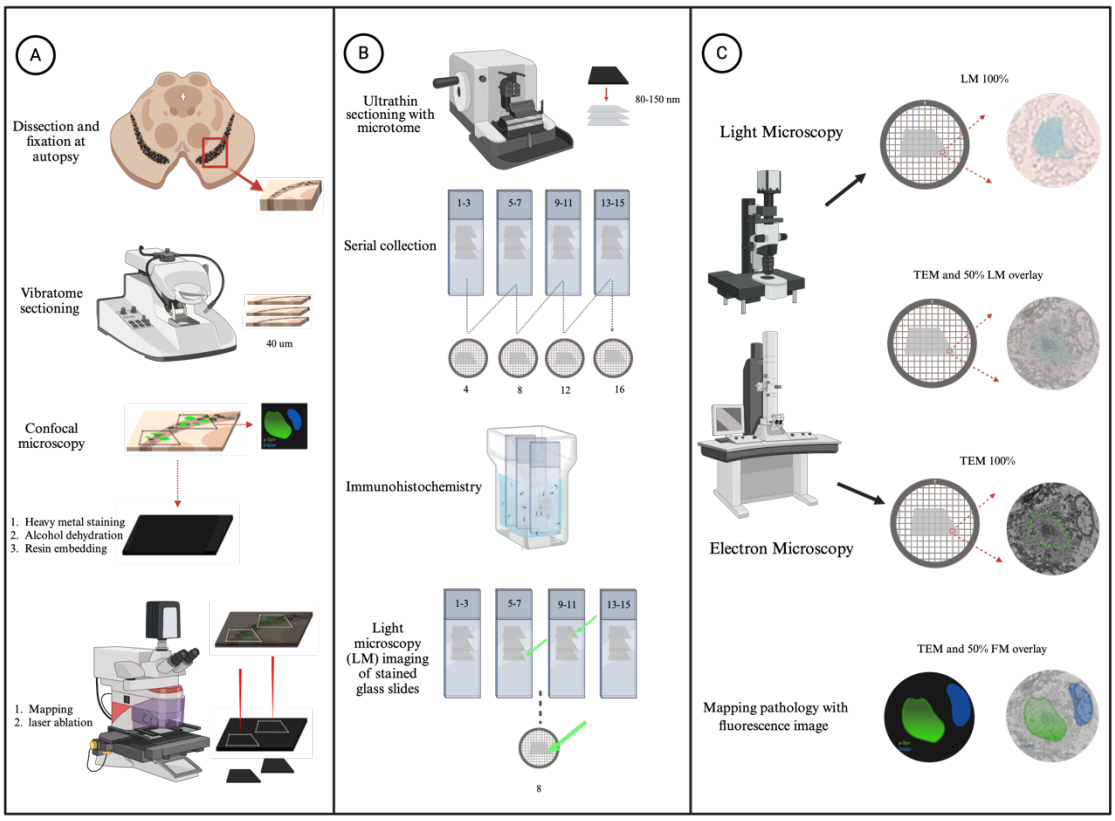

Figure 1. Schematic of the CLEM workflow; A) Brain samples are dissected and fixed at autopsy in 0.1% glutaraldehyde and 4% paraformaldehyde before being sectioned into 40 µm sections with a vibratome. The free-floating tissue sections are immunolabeled and imaged with fluorescence-based microscopy before being prepared for EM through *en bloc* staining and resin embedding. Blocks containing ROIs are excised from the tissue sections using laser ablation; B) The resin embedded and excised blocks are sectioned into 80-150 nm ultrathin sections which are serially collected on glass slides and EM grids. Immunohistochemistry staining is performed on the glass slides to identify the exact adjacent grid containing the ROI; C) The light microscopy images are used to guide the collection of high-resolution EM images and act as an intermediate bridge to correlate the fluorescence microscopy with the EM ultrastructure.

Sample preparation

Tissue fixation

We use post-mortem human brain tissue collected using a rapid autopsy protocol with less than 6 h post-mortem delay to limit the effects of tissue decay on the ultrastructure (Figure 2). The tissue is ideally fixed at autopsy in freshly made 4% paraformaldehyde (freshly dissolved in

buffer, or commercially available) and 0.1% glutaraldehyde in 0.1 M cacodylate buffer for 24 hours. We are aware of the high toxicity of the cacodylate buffer and its banning in many countries. In such cases, it can be replaced by phosphate buffer pH 7.4 for fixation[38]. For post-mortem human brain tissue we recommend the use of cacodylate buffer, not for its supposed effect of extraction but for its ability to efficiently neutralize free radicals in neural tissue in comparison to other buffers. According to our testing this is the optimal fixation method for preserving the tissue ultrastructure whilst retaining some tissue antigenicity (Figure 2). However, prospective rapid autopsy collection is not always achievable. Nonetheless, it is possible to maintain good tissue ultrastructure through post-fixation of an available paraformaldehyde-only fixed sample. In our experience the best ultrastructure will be maintained if the post-fixation in glutaraldehyde is carried out within 1 year of initial autopsy, with a gradual decline in ultrastructural integrity proportional to the amount of time spent in buffered formalin (formaldehyde and methanol in water) fixation.

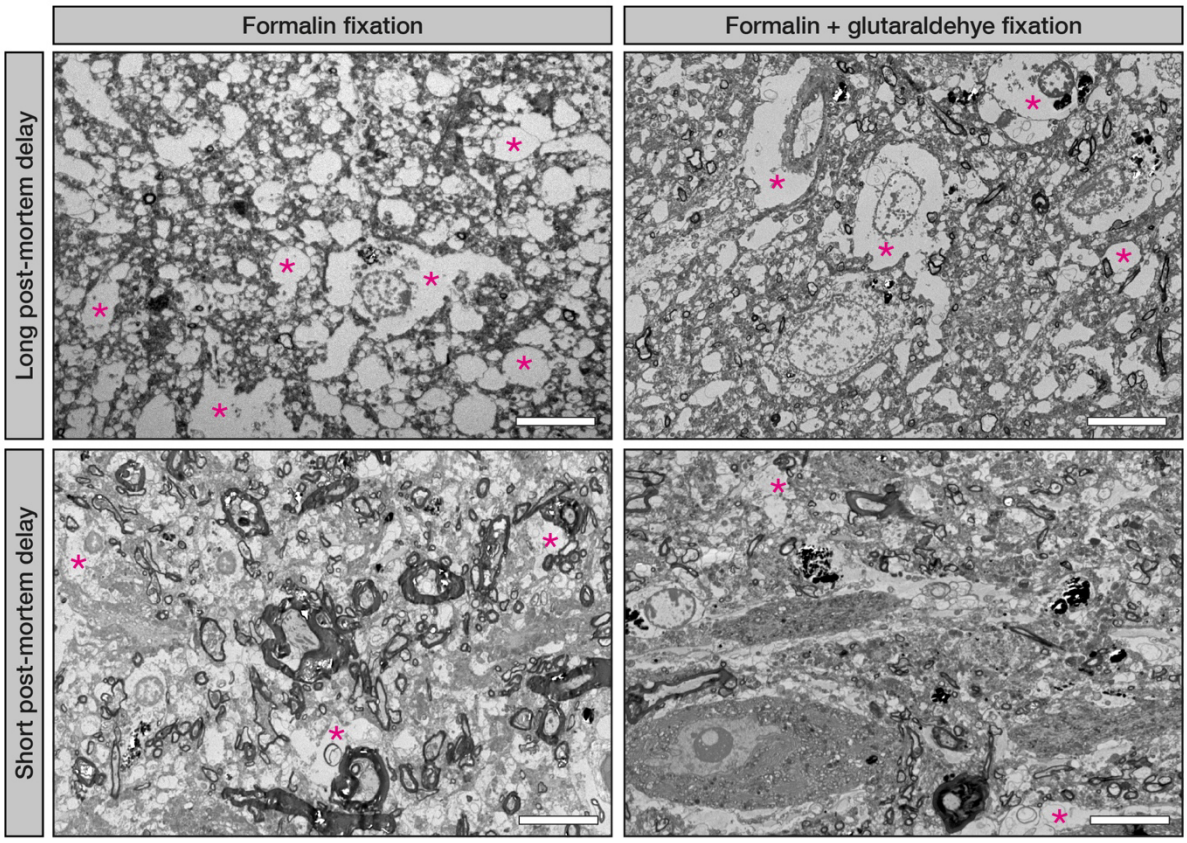

Figure 2. An example of the effects of post-mortem delay and chemical fixation on ultrastructure of human brain tissue. Tissue with a long post-mortem delay and fixed only in formalin result in large regions of empty space (magenta asterisks) due to the extraction of lipids and other cellular components. Samples fixed with glutaraldehyde show increased preservation of cellular material and combined with

a short post-mortem delay very little empty space is seen. Brain tissue from the *substantia nigra* collected by the Netherlands brain bank autopsy team with a post-mortem delay of 5 hours (short) or from the *cingulate gyrus* collected at the University Hospital Basel with a post-mortem delay of 96 hours (long) was chemically fixed in 4% formalin with or without 0.1% glutaraldehyde before identical sample processing for EM. Images were taken at room temperature on a T12 (FEI) electron microscope operated at 120 kV. Images adjusted for contrast. Scale bar is 10 µm.

Antibody labeling

The brain blocks are sectioned and stained with fluorescent dyes and antibodies to localize regions of interest in the tissue. Free-floating sections are incubated with primary antibodies followed by staining with secondary antibodies and dyes. To maintain ultrastructural integrity, we suggest avoiding permeabilization or antigen retrieval methods (e.g. detergents, picric acid, heat, formic acid). Chemical fixation creates holes in the membranes that allow many antibodies to penetrate, however the presence of glutaraldehyde can negatively impact the labeling. Multi-labelling can be used to tag the protein/structure of interest as well as the surrounding cellular context or physical tissue features which could be used later as fiducial markers. The number of labels that can be utilized is limited only by the number of channels available on the intended fluorescent microscope. DAPI penetrates glutaraldehyde-fixed tissue well up to at least 200 µm thickness, and we previously characterized several cell-specific markers[10]. The antibody concentration, staining time and tissue thickness should be optimized for each antibody used.

Fluorescent imaging

The free-floating sections are reversibly mounted on glass slides and imaged using fluorescence microscopy. The fluorescent labeling is used for mapping all ROIs in a given brain section (Figure 3). High resolution and volumetric z-stacks of the individual ROIs can be recorded here as well. The ROI map will be used again further in the protocol, after the resin embedding, to guide the precise excision of this region from the embedded sample. Alternatively, physical fiducial marks can be made in the tissue. However, we do not include a protocol for this in our pipeline. The fluorescent mapping protocol described here is optimized for free-floating sections, however a similar strategy could be applied to cell monolayers. For this application the cells should be grown on gridded coverslips for additional

physical fiducial markers[39]. Finally, our protocol describes the imaging of the sample by widefield fluorescence microscopy, however confocal or super-resolution microscopy (STORM, PALM, etc.) could also be used depending on the resolution and fluorescent characterization of ROIs required. Bleaching of the sample is not a limitation in this protocol as the fluorescence is not maintained after osmium impregnation and is not detected on EM sections.

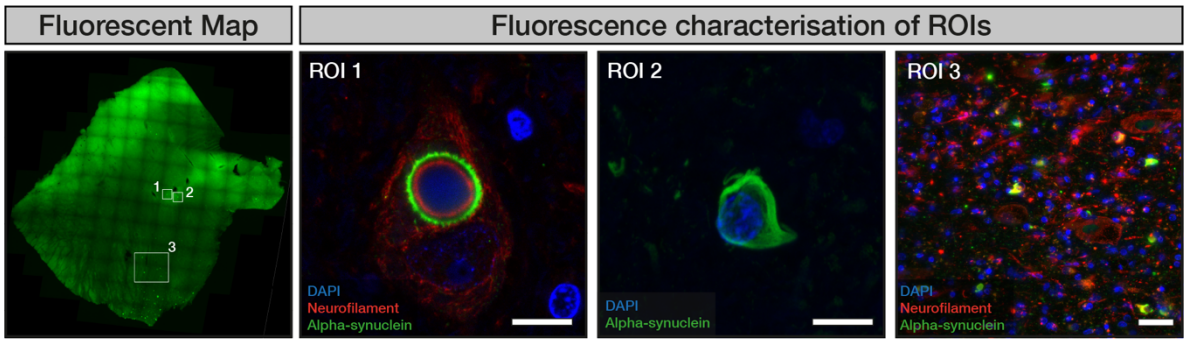

Figure 3. Fluorescent mapping of free-floating sections. A fluorescent overview of an entire brain slice is taken at low magnification. Individual cells or areas (white square) to be targeted for EM are annotated as accurately as possible on the overview image. High resolution images and/or volumetric z-stacks of the annotated ROIs can be obtained. ROIs 1-3 depict alpha-synuclein disease pathology in order: a single neuron containing a Lewy body, the pathological hallmark of Parkinson's disease; a single glial cytoplasmic inclusion, the pathological hallmark of multiple systems atrophy; a large region showing multiple alpha-synuclein inclusions alongside neurons from the brain of a multiple systems atrophy patient. For each image, the maximum fluorescence projections of volumetric z-stacks are shown, deconvoluted using the THUNDER algorithm (Leica). DAPI staining for cell nuclei plus antibody-specific immunostaining as stated are shown. Scale bar is 10 µm for ROI 1 and ROI 2, and 20 µm for ROI3. Images adjusted for contrast.

En bloc staining and resin embedding

After finding and mapping the ROIs on the sample, the section is stained with heavy metals, dehydrated and embedded in resin, in preparation for imaging by TEM. The *en bloc* staining protocol is based on published protocols without any significant modifications (rOTO + uranyl acetate + lead aspartate)[27–29]. Performing the complete protocol as written will provide high contrast and is recommended when SBF-SEM and FIB-SEM would be a potential downstream application for the sample. A shorter and more classic protocol (reduced Osmium - Osmium or

only reduced Osmium) can be used when the downstream application is TEM only. Depending on which staining steps are omitted the resulting EM grids may require post-staining with uranyl acetate (UA) and/or lead citrate to improve the contrast. For cell monolayers grown on coverslips the short protocol is recommended. In all cases, flat embedding is required in order to be able to image the sections by light microscopy.

We have described the protocol for manual *en bloc* staining and resin embedding of the samples, performed in glass vials or well-plates (Figure 4A,B). However, we have also optimized the protocol for use in an automated tissue processor (LeicaEM TP); (Supplementary Information). Large/thick samples (e.g. skin biopsies) can be housed in the standard Leica processor baskets provided (Figure 4C), however for vibratome sections of 40-150 micrometers, (e.g. human and mouse brain sections) we custom-designed a sample holder for the Leica EM TP comprising flat-discs spaced by 200 micrometers to stop the sections from excessive movement and curling/folding (Figure 4D). This sample holder can be built in-house on a 3D printer (however we recommend professional machining in surgical metal) by using the schematics and material information provided (Supplementary Figure 2-3).

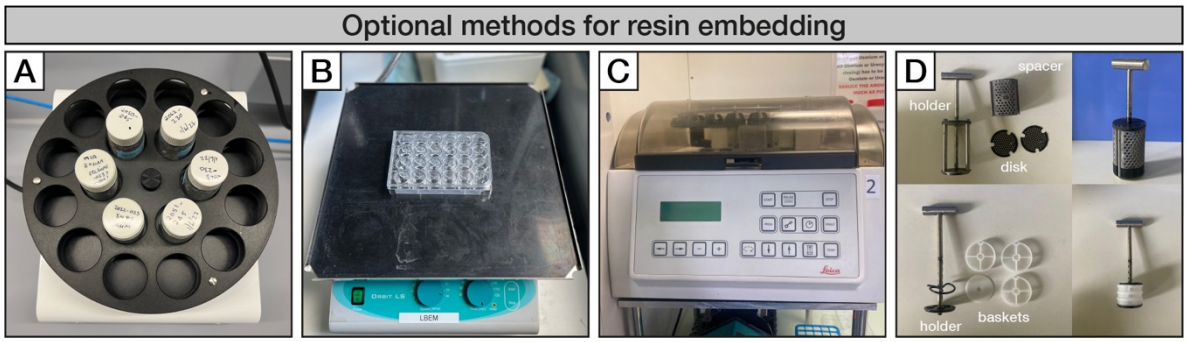

Figure 4. Methods for staining and resin embedding. The samples can be manually stained and resin embedded in glass vials with agitation by a rotator device (A) or in multi-well plates with agitation on an orbital shaker (B). Automatic staining and embedding can be performed in a tissue autoprocessor (C) in the supplied baskets for large samples, or a custom-made sample disks for free-floating sections (D).

Finding the region of interests

Preparation of blocks for CLEM sectioning

The regions in the sample containing the ROIs must be excised out of the flat embedded sample and glued on an empty resin block to be clamped in the ultramicrotome sample holder in preparation for serial sectioning. The embedded sections are first imaged with brightfield microscopy and then overlaid with the previously acquired fluorescence maps to mark the areas which need to be excised (Figure 5). The BigWarp [40] plugin in FIJI [41] can be used for a more precise alignment of the two images.

The excision of blocks is optimally performed with the use of lasers (e.g. laser capture microdissection) to provide maximal precision. However, if such equipment is not accessible, the blocks can be excised manually with a razor blade or scalpel, albeit with lower precision. Physical marks in the free-floating sections could be used for accuracy during manual excision. Alternatively, if the short staining protocol is used, some macro-features in the tissue still can be distinguished (arteries, large myelin tracts, different tissue densities etc) and used as fiducial markers (Figure 5). The area of the block that to be excised is up to the user's discretion, however, must be maximally limited in the X and Y dimensions to the area that will fit on the EM grids.

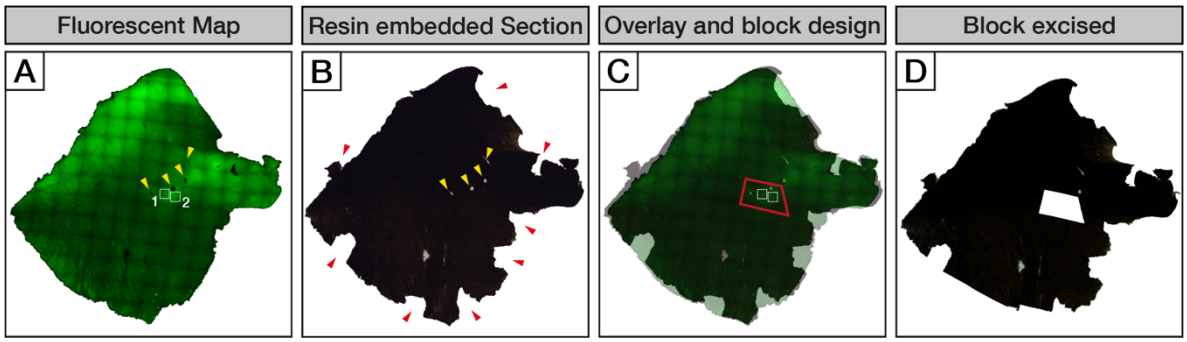

Figure 5. Preparation of blocks for CLEM sectioning. A brightfield image of the resin embedded section (A) is shown next to the fluorescent map made from the free-floating section (B). The edges of the resin section have been broken during the embedding process (red arrows) however, macro features visible in both images (yellow arrows) can be used for a precise alignment around the position of the ROIs (white circles). (C) The two images are overlaid, and a trapezoid shape (red outline) is drawn around the ROIs. (D) trapezoid block is excised from the resin-embedded section. Images were taken on a THUNDER Imager 3D Tissue (Leica) equipped with a K8

fluorescent camera (fluorescence) or a DMI8 colour camera (brightfield). Outline of the sections were digitally excised from the raw images for better alignment.

CLEM sectioning and immunohistochemistry (IHC)

Following the excision of the blocks from the embedded tissue, the blocks are mounted on resin blocks in preparation for CLEM sectioning (Figure 6). In this step we sequentially cut thin (<100 nm) and semi-thin (200 nm) sections with a Diatome Ultra diamond knife using an ultramicrotome and collect them alternatively on EM grids and glass slides, respectively (Figure 6). Immunohistochemistry staining (IHC) staining is performed on the glass slides where antibodies, visualized with a coloured dye, are used to target proteins of interest with the aim of identifying protein aggregates, cell types, or pathological features (Figure 6). Antigen retrieval can be performed on these sections as they won't be used for ultrastructural imaging. Additional antibodies that were not able to be included in the fluorescence can be used here, and several antibodies can be used during this step through multiplexed antibody labeling and dual chromogenic detection systems, or by collecting sections on multiple adjacent glass slides. Additionally, the slides can be stained with toluidine blue dye which offers higher contrast of the tissue in comparison to the IHC and allows the observation of morphological details comparable to that obtained by TEM at low magnifications (Figure 6). The slides are imaged by brightfield microscopy to locate the ROI within the sections and to follow the morphology of the tissue for later correlation with fluorescence and/or EM.

The alternate collection of serial sections on glass slides and EM grids is a key step in this technique, as it allows for the relocalization of the ROI within the embedded volume, the correlation of low-resolution light microscopy images with high-resolution electron microscopy images and acts as an intermediate bridge for the efficient correlation of fluorescence volume with the individual 2D ultrathin LM/EM images. The IHC step is useful for identifying which EM grid contains the ROI, and essential when fluorescence pre-localization has not been performed. However, toluidine blue staining alone can be used to correlate the fluorescence directly with the EM if the ROI is large and easily recognizable in the tissue[18].

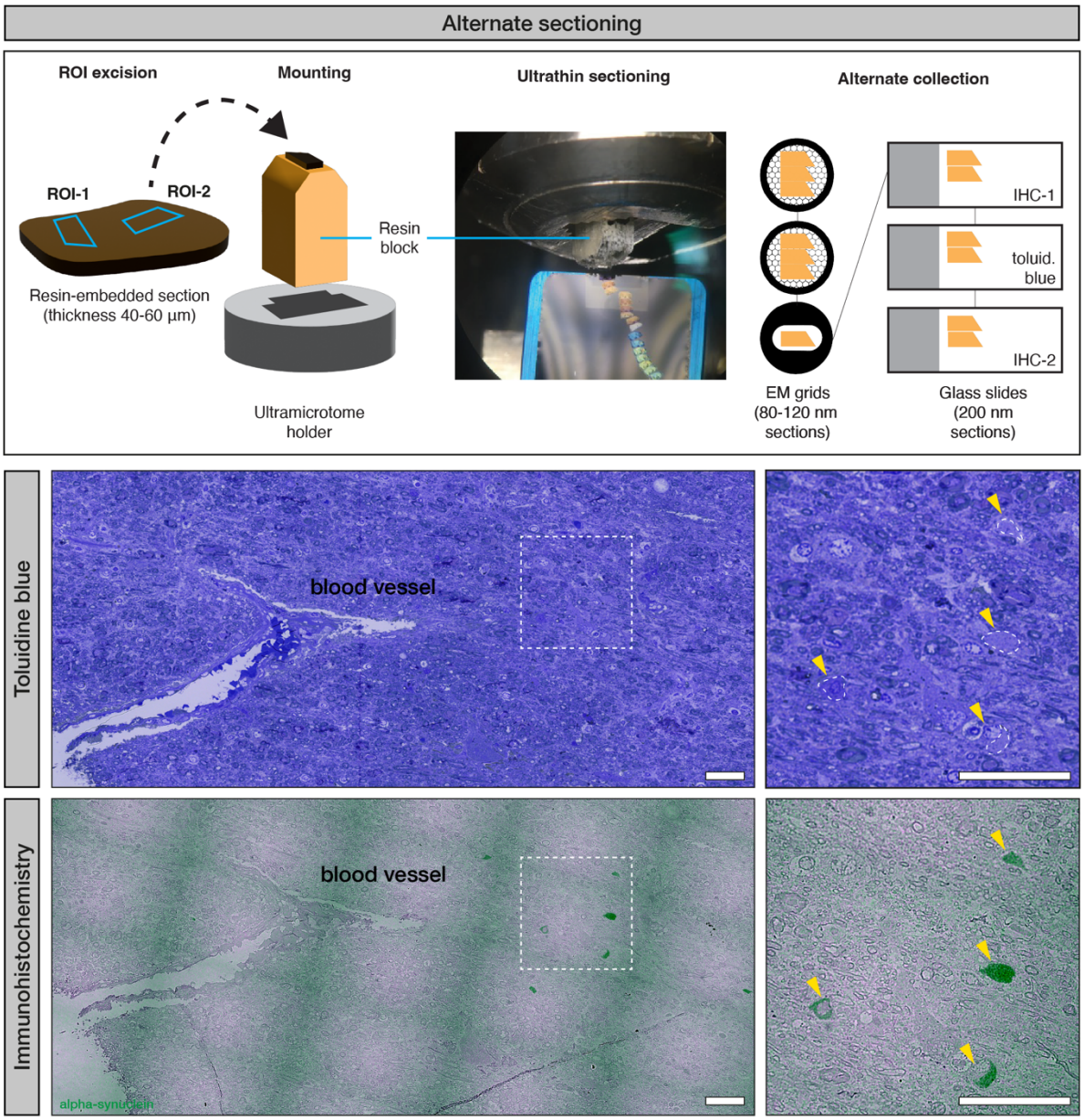

Figure 6. CLEM sectioning and IHC staining of brain tissue sections on glass slides. Blocks are excised from the resin-embedded section and mounted onto resin stubs. Serial ultra-thin sections (80-200 nm) are collected from the blocks. Sections of different thicknesses will refract different colours. The sections are collected alternatively on EM grids and glass slides in cycles all the way through the sample. The glass slides can be stained with IHC and toluidine blue to find which cycle contains the ROI (green). The IHC image is colour adjusted and the contrast strongly increased to increase visibility. Scale bars are 1 µm.

EM Imaging and Correlation

Correlating IHC and EM images

Bright field images of IHC sections are used to identify which cycles contain immunopositive ROIs. The comparison of tissue features and fiducials visible on both, EM images and bright field images of the same cycle, allows for the localization of ROIs on EM grids (Figure 7).
To facilitate this process, we wrote a python script (CLEM4CINA) that gives the TEM stage position by correlating sets of XY coordinates on the LM images with XY coordinates on the EM grid (Supplementary Information). This software is especially useful when the localization of ROIs by eye is difficult in crowded ultrastructural landscapes such as post-mortem human brain tissue.

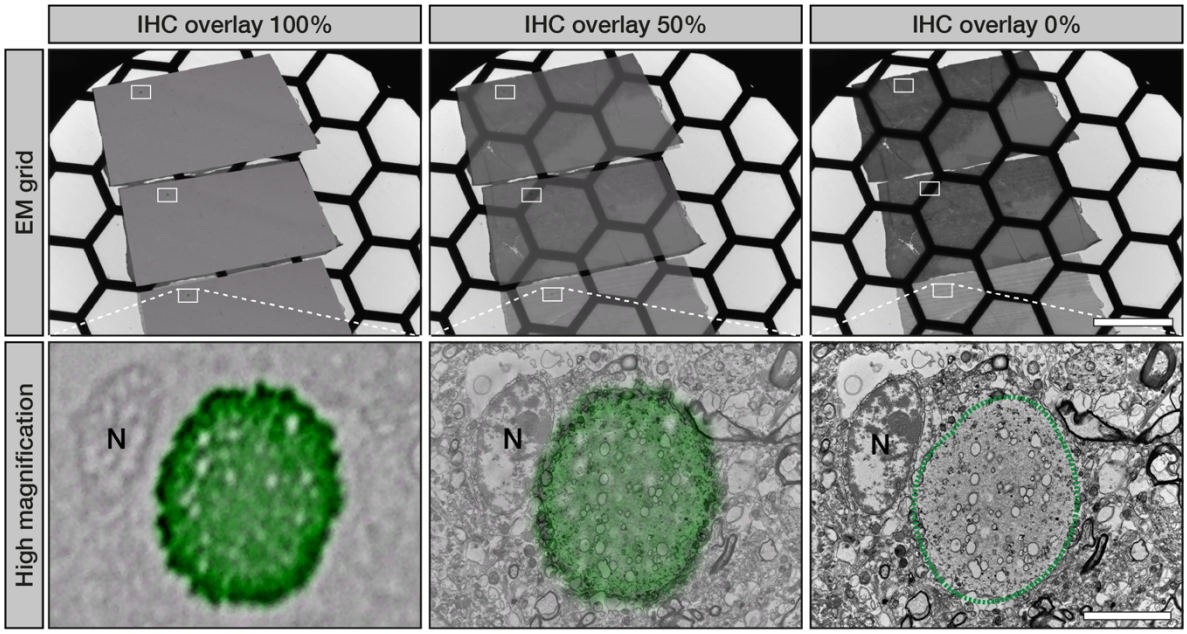

Figure 7. IHC and EM correlation. An ROI is located on the EM grid by overlaying a low magnification immunopositive brightfield IHC image with an overview of the serial sections on the EM grid within the same cycle. At higher magnifications, features visible in both imaging modalities, such as cell nuclei (N), can be used for exact immunopositive (green) ROI correlation. EM images were taken at room-temperature on a Tecnai G2 Spirit (ThermoFisher) operated at 80kV and equipped with a EMSIS Veleta camera. Scale bar is 250 µm for EM grid overviews, and 5 µm for high magnification images.

Correlating fluorescent and EM images

Throughout the CLEM procedure, the same sample has been imaged in different modalities, typically using (1) immunofluorescence, (2) IHC, (3) toluidine blue, and (4) EM. The direct correlation between fluorescent microscopy and EM images can pose a major challenge as the two imaging modalities greatly differ in size (two orders of magnitude in width and length, three orders of magnitude in depth). Further, the ultrathin IHC images have low tissue contrast, which can make it difficult to identify characteristic features. Therefore, we often use toluidine blue staining (which lacks immunopositive staining but has great tissue contrast), to aid the correlation between immunofluorescence, IHC, and EM. Toluidine blue sections are collected in the same manner as IHC sections and so cell nuclei, myelin sheaths and blood vessels, which are clearly visible, serve as landmarks that facilitate the image correlation (Figure 8). For image correlation, the interactive FIJI-plugin BigWarp[40] is used. This tool warps one (moving) image to another (target) image based on landmarks, i.e. user-defined points of correlation between both images.

Post-processing of a TEM tilt-series such as tomography and segmentation can be performed to obtain some 3D information, however, if the serial sectioning step is omitted, the same preparation protocol can be used to prepare samples for volume EM methods such as SBF-SEM or FIB-SEM.

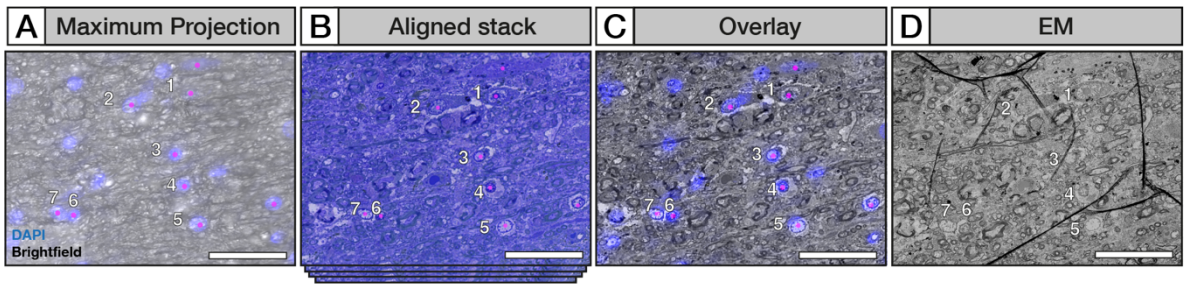

Figure 8. Fluorescence image and EM correlation. A) Fluorescence maximum projection of a 40 µm z-stack showing brightfield and DAPI channels (blue signal); B) After resin embedding and serial sectioning, the brightfield images of the 200 nm toluidine blue stained sections on the glass slides are aligned into a z-stack; C) Overlay of fluorescence maximum projection and toluidine blue stack (converted to black and white image) based on nucleus signal; D) The electron micrograph of a grid from the same cycle shown in A-C. The fluorescent maximum projection in (A) has been warped onto the toluidine blue image in (B) to identify exactly which grid should be imaged. Pink dots indicate cell nuclei in the fluorescence volume that could be correlated on the toluidine blue section shown in (B). The numbers indicate a nucleus pattern that is visible in fluorescence, toluidine blue images, and electron microscopy. Scale bar is 50 µm.

Expertise needed to implement the protocol

Our protocol assumes an understanding of basic wet-lab procedures and the concept of immunolabeling. In addition, researchers need to be experienced with vibratomes and ultramicrotomes for sample preparation, as well as brightfield, fluorescence, and electron microscopy for imaging. Users need to follow the safety rules and the regulations of their respective facility center when handling human brain tissue and all chemicals used in this protocol. Some familiarity with FIJI is helpful, but the publicly available software documentation should be sufficient to guide users through the use of this software for image correlation.

Limitations

We have used this CLEM pipeline to investigate subcellular ultrastructure within post-mortem human brain tissue[9–11,42], other human and mouse tissues (unpublished data, Supplementary Figure 1) and cell monolayers[11,16]. However, this protocol has several limitations that need to be considered. First, the native state of the sample will be altered due to the effects of chemical fixation, heavy metal staining and resin embedding. The specific changes due to these treatments have been well-documented elsewhere and will not be discussed here[17,28,43–46]. Additionally, the heavy metal staining, mainly used for generating contrast in the EM imaging, limits the achievable resolution to approximately 2 nm. Therefore, this protocol is unsuitable for atomic structural analysis of proteins or protein complexes. Generally, the thickness of EM sections is maximally limited to 200 nm. Thus, large volumetric information will not be obtained. Finally, the field of view is limited to the EM grid size (the maximum diameter is 3 mm). The protocol is not suitable for high-throughput EM analysis as it is time-consuming, labor-intensive, and requires careful coordination between multiple steps. However, depending on the ROI distribution in the sample, it may be possible to obtain all the information necessary from only a few samples. Furthermore, limitations may arise with antibody usability in EM fixed tissue, as well as the antibody penetration depth[10,47].

Materials

Reagent

Sample Fixation, vibratome sectioning and fluorescence labeling

- Paraformaldehyde 16% aqueous solution EM grade (Electron Microscopy Sciences, cat. no. 15700)

  CAUTION- Paraformaldehyde is harmful if swallowed or inhaled. It may irritate the skin. Wear suitable clothing, gloves and eye protection and work under the fume hood.

- Glutaraldehyde 25% aqueous solution EM grade (Electron Microscopy Sciences, cat. no. 16200)

  CAUTION- Glutaraldehyde irritates the skin. Wear suitable clothing and gloves

- Sodium cacodylate (Sigma-Aldrich, cat. no. C0250)

  CAUTION- Sodium cacodylate is irritant and toxic if swallowed. Wear suitable clothing and gloves.

  This chemical is banned by many countries and institutions. It can be replaced by phosphate buffer for fixation

- Glycerol (Sigma-Aldrich, cat. no. 1.04095)

- Hydrochloric acid (HCl, sigma-Aldrich, cat. no. 1.00317)

  CAUTION- Hydrochloric acid burns the skin and damages the eyes. Wear suitable clothing, gloves, eye protection and work in a fume hood.

En bloc staining and resin embedding

- Distilled deionised H2O (ddH2O)
- Calcium chloride (Sigma-Aldrich, cat. no. C4901)
- Acetone 99.5% (Acetone, Sigma Aldrich, cat. no. 32201-1L)

CAUTION- Acetone is flammable and toxic. Keep them in an allocated cupboard. Wear suitable clothing and gloves.

- Ethanol (Ethanol, Fischer Chemical, cat. no. E/0650DF/15)

  CAUTION- Ethanol is flammable and toxic. Keep them in an allocated cupboard. Wear suitable clothing and gloves.
- L-aspartic acid (Sigma-Aldrich, cat. no. 11189)
- Lead nitrate (Sigma-Aldrich, cat. no. 228621)

  CAUTION- Do not breathe the lead nitrate dust and avoid contact with skin or eyes. Wear suitable clothing and gloves and work in a fume hood.

- Osmium tetroxide 4% Aqueous solution (Electron Microscopy Sciences, cat. no. 19160)

  CAUTION- Osmium tetroxide is toxic if inhaled, comes into contact with skin and if swallowed. Wear suitable clothing, gloves and eye protection and work in a fume hood.

- Potassium ferrocyanide (Sigma-Aldrich, cat. no. P3289)

  CAUTION- Potassium ferrocyanide is irritant. Wear suitable clothing and gloves.

- Durcupan resin: supplied in four separate parts (Sigma-Aldrich, Ducupan Resin A/M, cat. no. 44611; Sigma-Aldrich, B hardener, cat. no. 44612; Sigma-Aldrich, D hardener cat. no. 44614; Electron Microscopy Sciences, DMP 30 cat. no. 13600)

  CAUTION- Durcupan resin is toxic by inhalation, in contact with skin and if swallowed. Wear suitable clothing, gloves and eye protection.

- Sodium cacodylate (Sigma-Aldrich, cat. no. C0250)

  CAUTION- Sodium cacodylate is irritant and toxic if swallowed. Wear suitable clothing and gloves.

- Sodium hydroxide (NaOH, Sigma-Aldrich, cat. no. 221465)

  CAUTION- Sodium hydroxide causes severe burns. Avoid contact with skin and eyes. Wear suitable clothing, gloves and eye protection.

- Thiocarbohydrazide (Sigma-Aldrich, cat. no. 223220)

CAUTION- Thiocarbohydrazide is toxic if inhaled, comes into contact with eyes and skin and if swallowed. Wear suitable clothing, gloves and eye protection.

- Uranyl Acetate (Electron Microscopy Sciences, cat. no 22400)

  CAUTION- Uranyl Acetate is radioactive material. When dealing with radioactive material, appropriate safety precautions must be followed.

Serial sectioning

- Distilled deionised H2O (ddH2O)
- Ethanol (Fischer Chemical, cat. no. E/0650DF/15)

  CAUTION- Ethanol and acetone are flammable and toxic. Keep them in an allocated cupboard. Wear suitable clothing and gloves.

- Borax (Electron Microscopy Sciences, cat. no. 21130)
- Toluidine Blue (Electron Microscopy Sciences, cat. no. 22050)

  CAUTION- Toluidine Blue is toxic if inhaled. Wear suitable clothing, gloves and eye protection.

Immunohistochemistry (IHC)

- Distilled deionised H2O (ddH2O)
- Potassium hydroxide (KOH, Sigma-Aldrich, cat. no. 1.05033.0500)

  CAUTION- Potassium hydroxide irritates and burns the skin. It is toxic if swallowed. Wear suitable clothing, gloves and eye protection.

- Triton X-100 (Sigma-Aldrich, cat. no. T9284)

  CAUTION- Triton X-100 is harmful when it comes into contact with the eyes and skin. Wear suitable clothing, gloves and eye protection.

- Hydrogen peroxide (H2O2, Sigma-Aldrich, cat. no. 1.07210.0250)

  CAUTION- Hydrogen peroxide can cause irritation to the eyes and skin. Wear suitable clothing, gloves and eye protection.

- Methanol 100% (Sigma-Aldrich, cat. no. 34860-1L-R)

  CAUTION- Methanol is flammable and toxic. Keep them in an allocated cupboard. Wear suitable clothing and gloves.

- Ethanol 100% (Fischer Chemical, cat. no. E/0650DF/15)

  CAUTION- Ethanol is flammable and toxic. Keep them in an allocated cupboard. Wear suitable clothing and gloves.

- Antibody diluent (Agilent Dako REAL, cat. no. S202230)
- Primary antibody (specific to protein of interest)
- Secondary antibody (according to the primary antibody species. Anti-mouse, Reactolab SA, cat. no.MPX-2402-15; Anti-rabbit, Reactolab SA, cat. no. MP-7401-15; Anti-goat, Reactolab SA, cat. no. MP-7405-15)
- HRP-green chromogen kit (Zytomed Systems, cat. no. ZUCO70-100)
- Mounting solution, NeoMount (Sigma-Aldrich, cat. no. 109016)
- Sodium chloride (NaCl - Fisher Chemical, cat. no. S/3160/60)
- Disodium hydrogen phosphate (Na2HPO, Sigma-Aldrich, cat. no. S9763)
- Potassium chloride (KCl, Roth, cat. no. 6781.1)
- Potassium phosphate monobasic (KH2PO4, Sigma-Aldrich, cat. no. P0662)

  CAUTION- Do not breathe the potassium phosphate monobasic dust and avoid contact with skin or eyes. Wear suitable clothing and gloves.

- (OPTIONAL) Formic acid (Sigma-Aldrich, cat. no. F0507)

  CAUTION- When using formic acid, it should be performed under a fume hood due to its irritants and burning effects on the skin and eyes.

- (OPTIONAL) Tris (Sigma-Aldrich, cat. no. 93352)
- (OPTIONAL) Ethylenediaminetetraacetic acid (EDTA, PanReac AppliChem, cat. no. A5097,0500)
- (OPTIONAL) Sodium citrate (Sigma-Aldrich, cat. no. 71402-250g)
- (OPTIONAL) Citric acid (Sigma-Aldrich,cat. no. C1909)

  CAUTION- Citric acid is irritant. Wear suitable clothing and gloves.

Reagent Setup

3 mM Calcium Chloride solution

- Dissolve 1.1 g of Calcium Chloride to 10 ml of ddH2O.

0.3 M Cacodylate buffer

- Dissolve 12.84 g of sodium cacodylate to 180 mL of ddH_2O and adjust the PH to 7.4 with HCl/NaOH, supplement with 3 mM of calcium chloride (600 µL) and make up the final volume to 200 mL with ddH_2O.

  CRITICAL- Sodium cacodylate is optimal for the preservation of membrane ultrastructure in neural tissue. Phosphate-based buffers can be used instead for fixation however rinsing steps have to be carefully performed to avoid precipitates

4% Paraformaldehyde, 0.1% glutaraldehyde in 0.15 M Cacodylate buffer as EM fixation buffer

- Mix 2.5 mL of 16% paraformaldehyde and 40 µL of 25% glutaraldehyde with 5 mL of 0.3 M cacodylate buffer and make up the final volume to 10 mL with ddH_2O.

  CRITICAL- Use a freshly opened vial of glutaraldehyde.

0.1% Paraformaldehyde in 0.15 M Cacodylate buffer as storage buffer

- Mix 62.5 µL of 16% paraformaldehyde with 5 mL of 0.3 M Cacodylate buffer and make up the final volume to 10 mL with ddH_2O.

10 M Tris-buffered saline (TBS)

- Dissolve 24 g of Tris Base and 88 g of NaCl in 900 mL ddH_2O water. Adjust pH to 7.4 with 1 M HCl and and make up the final volume to 1 L with ddH_2O.

50% Glycerol solution in TBS for fluorescence imaging

- Mix 1 ml of Glycerol with 1 ml TBS buffer.

Aspartic acid for *en bloc* staining and resin embedding the tissue

- Weigh out 0.2 g aspartic acid and add to 50 mL ddH_2O. Leave in a 60°C oven overnight to dissolve completely before use. The volume of solution prepared should be prepared 2-3 times in excess of what is needed.

2% Aqueous osmium tetroxide for *en bloc* staining and resin embedding the tissue

- Add 5 mL of 4% aqueous osmium tetroxide to 5 mL ddH_2O

Graded alcohol series (20%, 50%, 70%, 90%, 100%)

- Prepare 10 mL each of the indicated percentages of ethanol or acetone in ddH_2O.
  CRITICAL- If using a well-plate make sure the plastic is resistant to acetone or use ethanol for dehydration.

2% Reduced osmium for *en bloc* staining and resin embedding the tissue

- Add 2 mL of 0.3 M cacodylate buffer to 0.15 g of potassium ferrocyanide powder into a weight boat and pour into a 15 mL conical tube. Vortex well to dissolve before adding 5 mL of 4% aqueous osmium. Top up to a total volume of 10 mL with 0.3 M cacodylate buffer. The final concentration of the solutions should be 2% osmium tetroxide, 0.15 M cacodylate buffer and 0.15% potassium ferrocyanide.

Durcupan resin for *en bloc* staining and resin embedding the tissue

- Weigh out 10 g each part A and B with 0.3 g part D into a plastic wide mouth bottle. Mix thoroughly by stirring slowly on a magnetic stirrer. Add 16 drops of DMP-30 and mix thoroughly for another 10 minutes. The mixture turns red upon adding DMP-30. Keep stirring slowly until use but try to avoid air bubble formation.
  CRITICAL- Resin should be prepared fresh immediately before use.
  CAUTION- Resin should be prepared and used in a fume hood at all times. Excess resin should be polymerized in the wide mouth bottle in a 60°C oven for 48 hours before discarding.

1 M of sodium hydroxide (NaOH)

- Add 2 g of NaOH to 50 mL ddH_2O and agitate to dissolve.

1% Thiocarbohydrazide (TCH) for *en bloc* staining and resin embedding the tissue

- Add 0.1 g thiocarbohydrazide to 10 mL ddH_2O and place in a 60°C oven for 1 hour, mixing by inversion every 10 minutes. Cool down to room temperature and filter with a 0.22 µm syringe filter before using.

  CRITICAL- Some batches of TCH can produce a purplish-black solution after dissolving at 60°C, rather than being colourless. However, this does not affect the samples.

2% Uranyl Acetate for *en bloc* staining and resin embedding the tissue

- Add 0.2g of uranyl acetate powder to 10mL ddH_2O and agitate gently. Leave at room temperature to dissolve completely. Filter through a 0.22 µm syringe filter before use.

  CAUTION- Uranyl acetate is radioactive. Follow internal safety rules for radioactive materials.

0.66% Waltons Lead aspartate for *en bloc* staining and resin embedding the tissue

- Add 0.066 g lead nitrate per 10 mL of 60°C aspartic acid and mix by inversion. Keep the solution in a prepared 60°C water bath. Adjust pH to 5.5 (at 60°C) drop wise with fresh 1 M NaOH. Stabilize at 60°C for 30 mins.

  CRITICAL- This step is prone to precipitation if the temperature drops below 60°C, and/or if the NaOH is not fresh enough. The pH of the solution should be adjusted while the temperature is being maintained in the 60°C water bath. The pH of the solution changes quickly above pH 4 so the NaOH should be added dropwise with thorough stirring/swirling in between. In case the pH of the solution reaches above 5.5, it should not be corrected by the addition of acid. The solution must be prepared again from the beginning using the excess 60°C aspartic acid.

1% Toluidine blue solution

- Dissolve 0.1 g borax in 10 ml ddH2O. Add 0.1 g toluidine blue powder in 1% borax solution.

  CRITICAL- Store in a 10 ml syringe with a 0.22 µm sterile syringe filter at room temperature (RT).

CAUTION- Have a dedicated waste for toluidine blue. It is a strong dye, use ethanol to clean surfaces if spilled.

Saturated potassium hydroxide (KOH) using as etching solution

- Stir 11g KOH in 50 ml ethanol at RT overnight.

1x PBS, pH 7.4

- Mix 80.1g NaCl, 14.4g Na2HPO4, 2g KCl and 2.7g KH2PO4 to 800 mL ddH20. Adjust pH to 7.4 and make up the final volume to 1 L with ddH_2O.

20% Triton X-100 in PBS (PBS-T)

- Stir 500 ul of Triton X-100 in 200 ml PBS for at least 1 h.

  CRITICAL- Pipette Triton-X 100 at a slow rate due to its viscosity.

(Optional) 1x Tris-EDTA

- Mix 1.21 g tris, 0.37 g EDTA to 800 mL ddH2O until dissolved. Adjust pH to 9.0 and make up the final volume to 1 L with ddH_2O.

(Optional) 1x Sodium citrate

- Mix 24.3 g sodium citrate dihydrate and 3.4 g of citric acid to 800 mL ddH2O until dissolved. Adjust pH to 6.0 and make up the final volume to 1 L with ddH_2O.

Equipment

Sample fixation, vibratome sectioning and fluorescence labeling

- Leica vibratome (VT1200S)
- Cyanoacrylate glue (Biosystems AG, cat.no 14037127414)
- Brushes
- Double-sided razor blades (Science Services, cat. no. E72000)
- Leica THUNDER Imager 3D Tissue equipped with Flexacam C1 camera
- Leica Laser Microdissection Microscope (LEIM11505151)

- SuperFrost Plus Adhesion slides (Epredia, cat. no. J1800AMNZ)
- Precision cover glasses (Marienfeld, cat. no. 0107222)
- Microscope coverslips (Epredia, cat. no. BB02400240A153FST0)

En bloc staining and resin embedding

- Magnetic stirrer and stirring bars
- Orbital shaker (Labnet Orbit LS), vial rotator (Pelco R2 Rotary mixer) or tissue processor (Leica EM TP)
- Oven (Capable to reach 60°C)
- pH meter (with temperature probe highly advantageous)
- Water bath
- Weighing scales
- Aclar 33C Embedding Film (Electron Microscopy Sciences, cat. no. 50425-10)
- 15 and 50 mL centrifuge tubes (ThermoFisher Scientific, cat. no. 33.96.50; cat. no. 339652)
- Embedding squares for cell monolayers (Gene frames; ThermoFisher Scientific, cat. no. AB0577)
- Flat embedding molds (14mm x 5mm x 3mm, Electron Microscopy Sciences, cat. no. 70902)
- Glass scintillation vials with a poly-seal cone liner (Electron Microscopy Sciences, cat. no. 72632)
- Multi-well plates (12 wells, polystyrene plate, flat bottom. Sigma-Aldrich, cat. no. CLS3512)
- Tooth-picks / applicator sticks (Electron Microscopy Sciences, cat. no. 72304-10)
- 20 ml, 10 ml and 5 ml syringes (VWR, cat. no. 720-2521; cat. no. 720-2520; cat. no. 720-2519)
- Sterile Syringe Filters 0.22 μm (Merck, cat. no. SLGV013SL)
- Plastic wide mouth bottle (Fisherbrand, cat no.02-896B)

Serial sectioning

- Ultramicrotome (Leica, EM UC7)
- Diamond knife (Ultra 45°, Diatome, cat. no. DU4515)

CAUTION- It is extremely important to keep diamond knives in a safe place. For the user and knife's safety, it is important not to touch the knife's tip.

- Trimming knife (Trim 90, Diatome, cat. no. DTB90)

  CAUTION- It is extremely important to keep diamond knives in a safe place. For the user and knife's safety, it is important not to touch the knife's tip.

- Digital dry bath (Labnet, Accublock Digital Dry Bath)

  CAUTION- Avoid touching the hot plate while it is on.

- 2 x Eyelash manipulator (Electron Microscopy Sciences, cat. no. 71182)
- Double-sided razor blades (Science Services, cat. no. E72000)

  CAUTION- Razor blade is sharp. Avoid touching the edge of the razor blade.

- Electron microscopy grids (Electron Microscopy Sciences, cat. no. FF-100H-Cu-UL, cat. no. FCF2010-CU-TA-50ST)
- Grid storage box (Electron Microscopy Sciences, cat. no. 71152)
- Superfrost plus glass slides (BioSystems, cat. no. 85-0915-00)
- Qualitative filter paper (Whatman, cat. no. 1001090)
- Diamond scribing tool (Electron Microscopy Sciences, cat. no. 70030)
- Super glue (Loctite Adhesive 410, Digitec Galaxus, cat. no. 6489185)
- Reverse action tweezer (Ideal-tek, cat. no. 3X.SA.1)
- Resin stubs previously prepared
- Loops (Electron Microscopy Sciences, cat. no. 70944)

Immunohistochemistry (IHC)

- Steamer (Tristar steamer 12 L)
- Oven (Capable to reach 37°C)
- Pathology pen (Agilent, cat. no. S2002002230-2)
- Staining tray (Fisher Scientific, cat. no. 155189960)
- Staining jars (Fisher Scientific Kartell, cat. no. 10375681)
- Glass staining jar (VWR, cat. no. 631-0698)

Other

- Transmission electron microscopy 80kV-120 kV
- Computer
- FIJI ([41]
- (OPTIONAL) Python compiler
- Fume hood

Procedure

Sample fixation

Timing: (a) 24.5 h, (b) 2.5 h, (c) 1.5 h

1. Fix the samples for EM according to the protocol for (a) post-mortem human brain, (b) mouse tissues or (c) cell monolayers grown on coverslips.
    a. Sample fixation for human brain:
        i. Dissect brain or tissue blocks based on anatomical landmarks, volume 0.5 mm^3.
        ii. Immerse the brain blocks in EM fixation solution for 24 h at 4°C.
        iii. Transfer the samples into the storage solution and keep at 4°C.

    NOTE- If the tissue blocks were not fixed for EM at autopsy, post-fix the tissue blocks in EM fixative, ON, 4°C.

    b. Sample fixation for mouse tissues:
        i. Anesthetize the mouse following local institutional guidelines. Perfuse the mouse via the heart with a perfusion pump in EM fixation solution at a speed of 7 ml/min using approximately 250-300 ml per animal.
        ii. After perfusion, leave the animal for 1 hr.
        iii. Dissect the relevant tissues according to local institutional guidelines.
        iv. Transfer the samples into the storage solution and keep at 4°C until use.
    c. Sample fixation for cell monolayers grown on coverslips:

i. Dilute the EM fixation solution 50% in cell-growth media/buffer.
ii. Exchange the cell-media with the diluted EM fixation solution and incubate for 20 mins at room-temperature.
iii. Exchange into undiluted EM fixation solution for 1 hr at room-temperature.
iv. Wash the cells with three exchanges of 0.15 M cacodylate buffer for 5 min each at room-temperature.
v. Proceed immediately to resin embedding protocol for cell monolayers (Steps 32-52).

Vibratome sections - Sample preparation

Timing: 30 mins per sample

2. Fill sample tray of the vibratome with 0.1 M cacodylate buffer.
3. Prepare a collection well-plate or sample vials containing cacodylate buffer

NOTE- If the free-floating slices will be immunolabeled and imaged with fluorescence the samples can be collected in TBS as this buffer has better spectral properties for imaging.

4. Put a small amount of cyanoacrylate glue on the sample plate of the vibratome.
5. Place the brain block on the glue. Nudge the block with a paint-brush after a few seconds to ensure it is stable.

CRITICAL- Sample should not dry out.

6. Immerse the sample plate into the sample tray containing the cacodylate buffer. The glue will harden upon immersion in the buffer.
7. Set amplitude and speed .
8. Set start and end points of the section.
9. Start cutting.

CRITICAL - Choose speed and amplitude that lead to consistent cuts. Decreasing speed and increasing amplitude allows cutting of softer tissue and thinner sections. The desired thickness depends on the scientific question and technical aspects (such as size of ROI, antibody penetration, flat embedding etc).

10. Use a brush to collect sections into storage vials/well plate containing storage buffer.
11. Remove sample tray and decant the cacodylate buffer with tissue debris into a prepared waste container (remove big pieces of brain if necessary).

CAUTION- Brain waste is considered as bio-hazardous. Follow the internal safety rules of your facility/institute regarding waste disposal.

12. Remove the leftover glued brain by carefully sliding a razor blade between the sample and the support.
13. Peel off the attached glue from the tissue and discard.
14. Put the leftover tissue back in the storage buffer.
15. Store vibratome sections in the collection buffer until use at 4°C.

Immunolabeling of free-floating sections

Timing: 3 hours to overnight (depending on antibody incubation time)

NOTE- Fluorescence staining may require pre-testing of antibody concentrations and incubation parameters. Antigen retrieval could be performed here however the effect on the ultrastructure would need to be carefully assessed.

16. Wash the free-floating sections with three exchanges of 1x TBS for 5 min each at RT on orbital shaker.
17. Prepare the primary antibody cocktail in 1x TBS.
18. Immerse the sections in the primary antibody cocktail. Use enough primary antibody to adequately cover the sections.
19. Agitate the samples on an orbital shaker for the desired incubation time.
20. Wash the free-floating sections with three exchanges of 1x TBS for 5 min each at RT on orbital shaker.
21. Incubate the sections in secondary antibodies coupled to fluorescent dyes for 1h at RT on an orbital shaker.
22. Wash the free-floating sections with three exchanges of 1x TBS for 5 min each at RT on orbital shaker.
23. Store the sections in 1x TBS at 4°C for imaging. CRITICAL- If samples are stored long term it is best to add 0.1% final

concentration of PFA to 1x TBS to preserve the ultrastructure (in analogy to the cacodylate storage buffer).

Fluorescent imaging and ROI mapping

Timing: Up to several hours per tissue section depending on the number of ROIs and size of area to be imaged.

This section includes a general outline with the key points needed for creating a fluorescent map to be used for later correlation.

24. Reversibly mount the section on a glass slide.
    a. Place a drop of mounting solution (50% Glycerol, 50% TBS) onto a glass slide
    b. Pick up a free-floating section using a brush and carefully roll the section off the brush and into the mounting medium on the glass slide
    c. Manipulate the section with the brush to ensure it is completely flat and devoid of wrinkles or folds.
    d. Place a precision coverglass directly on top of the section avoiding the creation of air bubbles over the sample.
    e. Use a tissue to wick away excess liquid and secure the coverglass onto the glass slide.
25. Insert the glass slide into the microscope slide holder.
26. Take a low magnification overview of the entire section (e.g. using a 10 x objective) in brightfield and fluorescence.
27. Image the ROIs at higher magnification if desired
    a. Adjust laser intensity, aperture and exposure to get an appropriate fluorescent signal for all channels at the magnification to be used for imaging.
    b. Determine upper and lower z values of the ROI.
    c. Record the z-stack.
28. Annotate the position of each imaged ROI as accurately as possible on the brightfield/fluorescent overview image.

NOTE- Some imaging softwares have integrated options to do this. Otherwise, the overview image can be opened in an external software and manually added.

29. Continue imaging and annotating ROIs as necessary.
30. Unmount the tissue section
    a. Immerse the glass slide in a 50 ml tube containing 1x TBS and wait for the coverslip to detach (~5 mins).

    NOTE: physically forcing the detachment of the coverglass is not recommended as the sections can easily tear.

    b. Retrieve tissue section with brush.
31. Store the section in 1x TBS ready for resin embedding.

PAUSE POINT- Sections can be stored in storage buffer at 4°C for a few weeks.

En bloc staining and Resin embedding

This section details the full protocol for staining and resin infiltration and flat embedding of free-floating vibratome sections and cell monolayers. Depending on the application a shorter protocol omitting some of the staining steps can be used. For SEM, the full protocol is recommended, the short protocol is recommended for TEM only and the preparation of cell-monolayers. For large samples (e.g. skin biopsies) the incubation times may need to be increased depending on the thickness of the sample.

Timing: Full protocol-24 hours split into 2-3 days or short protocol-1.5 days, plus 48 hours resin curing.

CAUTION- For the most consistent infiltration of solutions into the tissue, all steps should be performed with agitation unless otherwise stated.

CAUTION- This step requires many hazardous substances therefore all steps should be performed in a fume hood.

NOTE- Omit steps 33-36 and 39-44 for short protocol.

32. Wash the samples with multiple exchanges of cold 0.15 M CaCO buffer for a total of 15 minutes to remove all traces of chemical fixative (at least 3 exchanges of 5 mins each).
33. Post-fix the samples by incubation in reduced osmium for 1 hour on ice. (Can be included in the short protocol for better preservation of membranes)

CRITICAL- Prepare fresh TCH during this incubation step.

34. Wash the samples with multiple exchanges of ddH_2O at room-temperature for a total of 15 minutes (e.g. at least 3 exchanges of 5 minutes each).
35. Immerse the samples in filtered TCH for 20 minutes.

36. Wash the samples with multiple exchanges of ddH_2O at room-temperature for a total of 15 minutes (e.g. at least 3 exchanges of 5 minutes each).
37. Post-fix the samples by incubation in 2% Osmium tetroxide in ddH_2O for 30 minutes at room temperature.
38. Wash the samples with multiple exchanges of ddH_2O at room-temperature for a total of 15
39. Immerse the samples in 2% filtered uranyl acetate in ddH_2O and leave for 1 hour at room temperature

CRITICAL- Prepare aspartic acid for the following day.

PAUSE POINT- The samples can be left at 4°C overnight.

40. Wash the samples with multiple exchanges of ddH_2O at room-temperature for a total of 15 minutes (e.g. at least 3 exchanges of 5 minutes each)
41. Bring samples to 60°C by incubation in a water bath or oven for 30 minutes.
42. Prepare Waltons lead aspartate at pH 5.5, 60°C and stabilize at 60°C for 30 minutes in a water bath.
43. Immerse the samples in Waltons lead aspartate and incubate at 60°C for 40 mins without agitation.

CRITICAL- Prepare the Durcupan resin during this step.

44. Wash the samples with multiple exchanges of ddH_2O at room-temperature for a total of 15 minutes (e.g. at least 3 exchanges of 5 minutes each).
45. Dehydrate the samples using a graded alcohol series (20%, 50%, 70%, 90%, 3 x 100%) through immersion for 5 minutes each at room-temperature.

CRITICAL- Samples will become brittle after alcohol dehydration so care should be taken when handling them after this point.

46. Immerse the samples in 50% resin in alcohol for 30 minutes at RT.
47. Exchange the samples into 100% resin for 1 hour.
48. Exchange the samples into fresh 100% resin and leave to infiltrate overnight.

CRITICAL- It is critical that the samples are agitating well (but not to cause air-bubbles in the resin or cause the brittle samples to break) so they do not stick to the bottom of the vials/well plate during this long incubation period.

49. Prepare fresh Durcupan resin.
50. Exchange the samples into 100% resin and incubate for 2 hours.

NOTE- Use the excess resin to fill flat embedding molds to make resin stubs for mounting samples in preparation for ultramicrotomy.

51. Flat embed the samples

a. For Tissue slices

i. Prepare a glass-slide and Aclar sandwich (Figure 9A).

NOTE: Make sure the Aclar sheets are bigger in all dimensions than the glass slide to avoid the glass slides being stuck together by the cured resin.

ii. Place a drop of resin on one of the Aclar pieces.

iii. Use a toothpick or cut a diagonal surface on the end of an applicator stick to pick up and manipulate the samples in the resin.

iv. Pick up the slices and drop onto the Aclar film being careful not to break the samples.

v. Add some additional resin surrounding the sample.

NOTE- Don't add so much resin that it bleeds out over the edges of the Aclar when the sandwich is placed on top, but enough so that the Aclar sticks.

vi. Roll the second piece of Aclar film carefully against the tissue slices in a way that avoids air bubble formation.

vii. Sandwich the Aclar between two glass slides.

b. For cell monolayers

i. Place an embedding square that is smaller than the size of the coverslip on top of some Aclar film and fill with a thin layer of resin.

ii. Pick up the coverslip from the well-plate using tweezers and place cell-side down onto the embedding square ensuring it makes contact with the resin below (Figure 9B).

iii. Place flat in a 60°C oven to cure.

52. Cure the resin in a 60°C oven for 48 hours.

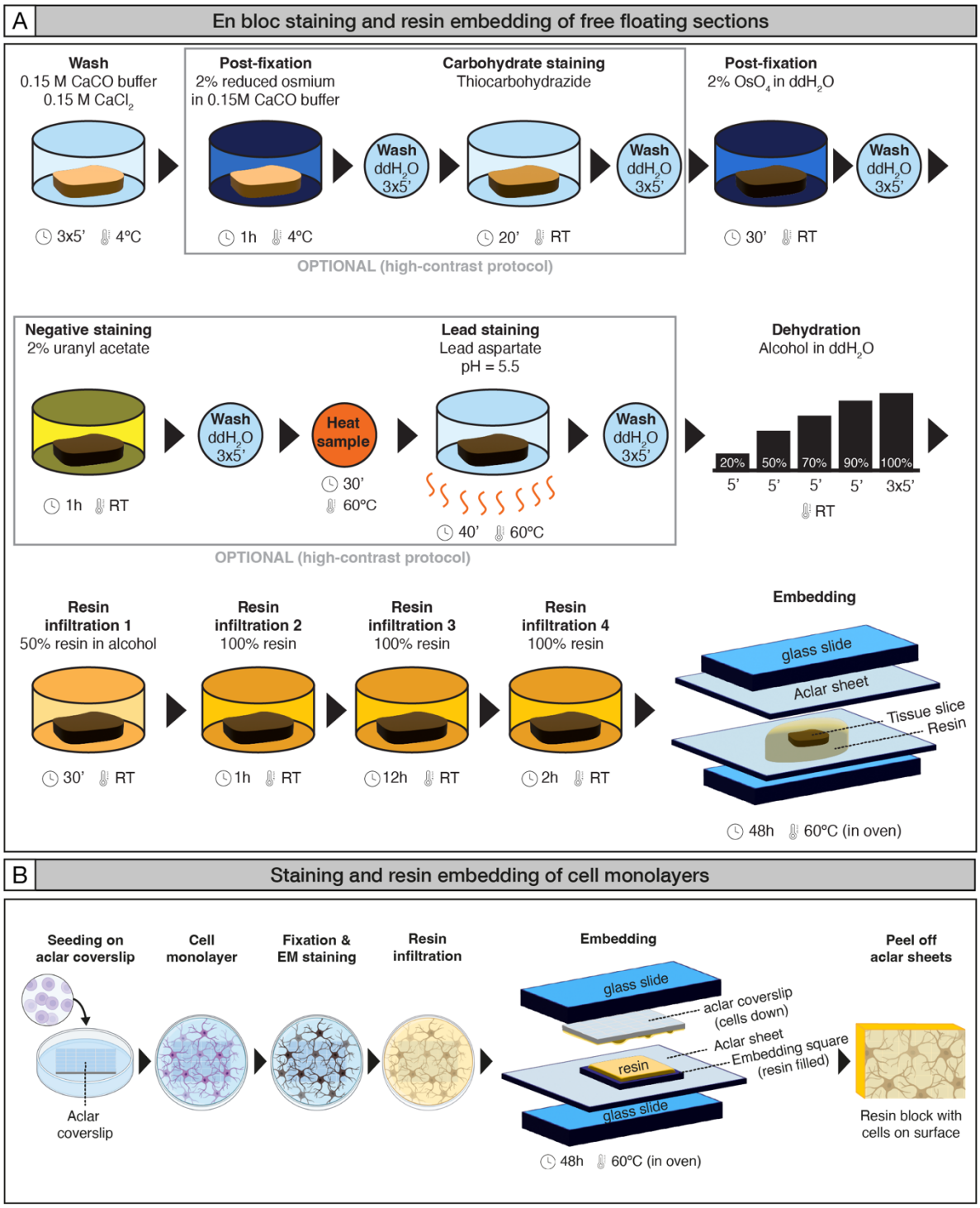

Figure 9. Schematic of resin embedding process. A) free-floating sections; B) cell monolayers grown on coverslips

Block preparation

Timing: 1-2 hours

53. The regions in the sample containing the ROIs must be excised out of the sample and mounted on a resin stub in preparation for serial sectioning. This can be done manually or by laser-capture microdissection (LMD) by following options a or b, respectively.
    a. Manual excision
        i. Use a razor blade/scalpel to excise the region of interest under binocular.
        ii. Mount on a resin block (steps 54-56).
        iii. Use a razor blade to trim the block surface area into a trapezoid shape and to fit on an EM grid.
    b. Laser-dissection
        i. Take a brightfield image of the entire section.
        ii. Overlay the image of the resin section with the fluoro/brightfield map in any suitable software (e.g. ppt/illustrato**r** etc. Figure 6).
        iii. Draw trapezoid shapes around the ROIs with an area that will maximally fit on a 1 x 2 mm slot grid (e.g. - 1.5 x 0.7 mm).

        NOTE- The trapezoid shape helps for sample orientation in the downstream EM imaging. Try to put ROIs in the center of the block away from the edge.

        iv. Transfer the position of the shapes according to the specifications of the laser microscope to be used for cutting.

        NOTE- The laser-microdissection microscope we use requires the import of user-designed shapes with the coordinates as a .xml file. We have included this protocol in the Supplementary Information.

        v. Laser cut the trapezoid blocks.
        vi. Remove laser cut shapes by placing sticky tape over the section and slowly peeling it back. The cut shapes should adhere easily to the tape and can then be mounted on resin blocks.
54. Spread super glue on top of an empty resin block.

NOTE- The surface of the block can be scratched with sandpaper for better adherence and to create a flat surface.

CAUTION- Resin particles created by sandpapering can be a skin irritant. Perform this step in a fume hood and wear protective clothing.

55. Pick up the excised block with a wetted toothpick and mount on top of the prepared empty resin block.
56. Wick away the excess glue taking care not to dislodge the sample.

CRITICAL- The glue must dry completely in order to ensure it is stable for ultramicrotomy. This step can be done the night before or a few hours prior to the cutting session.

Serial sectioning and collection

Timing: 2 hours per sample

57. Place the resin block on the sample holder and use a razor blade to trim away empty resin on the support resin block.
58. Place the sample on the ultramicrotome arm sample holder
59. Mount the diamond knife on the ultramicrotome.

NOTE- If there is too much excess resin on top of the sample, this can be first trimmed away using a trimming knife. The trimmed resin will look white on the knife. As soon as the knife reaches the sample in the resin block, the trimmed parts look slightly darker. However, caution should be used to ensure not too much sample is trimmed away in case the ROI is located at the top of the block in z-height.

60. Align the knife edge to the block surface.

CRITICAL- The correct alignment of the block to the knife is necessary to minimize sample and region of interest loss during the cutting.

61. Approach the diamond knife to the sample until the light reflection on the sample surface is barely seen.
62. Set the following parameters on the ultramicrotome:
    a. Cutting window (start/end cutting point)
    b. Sample thickness (variable (50-200 nm)
    c. Cutting speed (1.6 mm/s)
    d. Activate the counter mode to have an estimation of how much resin is cut

63. Overfill the boat of the diamond knife with filtered ddH2O to make sure the entire surface of the knife edge is wet.
64. Use a small syringe fitted with a hypodermic needle to remove excess water until the water surface becomes shiny and flat like a mirror.

CRITICAL- The diamond knife must always stay hydrated while cutting, otherwise the sections will shrink and fold.

65. Start cutting some sections to check if all the empty resin has been removed.

NOTE- Usually the resin-only section is very smooth and uniform while if it contains tissue some pattern is visible on the surface of the section and it can appear rough.

66. (OPTIONAL) To check whether the section contains any sample or if it is resin-only, collect 1-2 sections on a glass slide and stain with toluidine blue:
    a. Use the eyelash manipulators to isolate the section (s) of interest.
    b. Use the loop to pick up 1-2 sections.
    c. Place them on a clean glass slide.
    d. Let the water droplet dry by placing the glass slide on the hot plate.
    e. Cover the section (s) with a droplet of toluidine blue solution.
    f. Heat the slide on the hot plate (at roughly 100°C) for about 30 seconds, or until the droplet edge dries (turns green).
    g. Rinse with ddH2O until the droplet edge has been cleared.
    h. Dry the slide on the hot plate for a few seconds until the water evaporates.
    i. Inspect the slide with a light microscope. NOTE- If the section contains any sample, cellular and extracellular components will now be visible, otherwise the section will remain transparent (Fig 10A).
67. If necessary, repeat steps 66-67 until the sample or the region of interest becomes visible in the section.
68. When ready for collection prepare the following material:
    a. Use the diamond scribing tool to scratch the bottom surface of some glass slides in order to create reference positions, to later unambiguously identify the sections in the correct order (Figure 10B).

b. Prepare some tweezers with EM grids according to the collection strategy.

CRITICAL- The order of the sections during the collection might be very important so make sure to have all the tools ready and prepared for the serial collection.

69. Cut a ribbon containing 14 sections for one CLEM cycle as follows: 6 semi-thin sections (200 nm) for glass slides, 7 ultra-thin sections (<100 nm) for EM grids, 1 semi-thin section (200 nm) to be left on the glass knife for the next cycle.

NOTE- Sections of different thicknesses have different colours (blue/green - 200-250 nm, silver - 60-80 nm) (Figure 6). This is helpful to remember which sections need to be placed on glass slides and on EM grids. The collection as described here will span approximately 1.5 μm depth in 1 CLEM cycle. For collection all the way through a 20 μm ROI/cell, 18 CLEM cycles should be collected. The number and thickness of the collected sections can be changed according to the end-requirements. E.g. - thicker EM sections (120 nm) could be collected for tomography and less sections in the CLEM cycle for a smaller ROI.

NOTE- Consider collecting multiple glass slides replicates in parallel, in order to have multiple IHC labeling possibilities and/or to include toluidine blue staining on one slide

70. Transfer the sections on glass slides/grids in the correct collection order (Figure 10B).
    a. Use the eyelash manipulators to detach the ribbon from the knife leaving the last-cut semi-thin section on the knife, separate the section (s) of interest into groups as they will be collected on the glass slides and EM grids.
    b. Use the loop to pick up the first 3 sections and place them on the first glass slide in position 1.
    c. Use filter paper to wick away the water from the loop leaving the sections flat on the glass slide.
    d. Use the loop to pick up the second 3 sections and place them on the second glass slide in position 1.

e. Immerse an EM grid in the water bath and position it underneath a group of ultra-thin sections (for a mesh grid) or a single isolated section (for a slot grid)

f. Use the eyelash manipulators to slide the sections on the grids while simultaneously lifting the grid out of the water bath. CRITICAL- Sections (especially the thin ones) are very prone to breakage. Hold the tweezer with the grid with one hand and keep it underwater near the selected section and with the other hand use the eyelash manipulator to gently move and place it on the grid. Once the section is on the grid, move the tweezers out of the boat and let the water droplets on the grid dry.

71. Once the collection is finished, place the glass slide on the hot plate for 15 mins so that all the water dries out and the sections properly adhere to the glass slide.

72. Store all the grids in an empty grid box.

PAUSE POINT- The sections mounted on both the glass slides and EM grids can be stored indefinitely at room-temperature until needed.

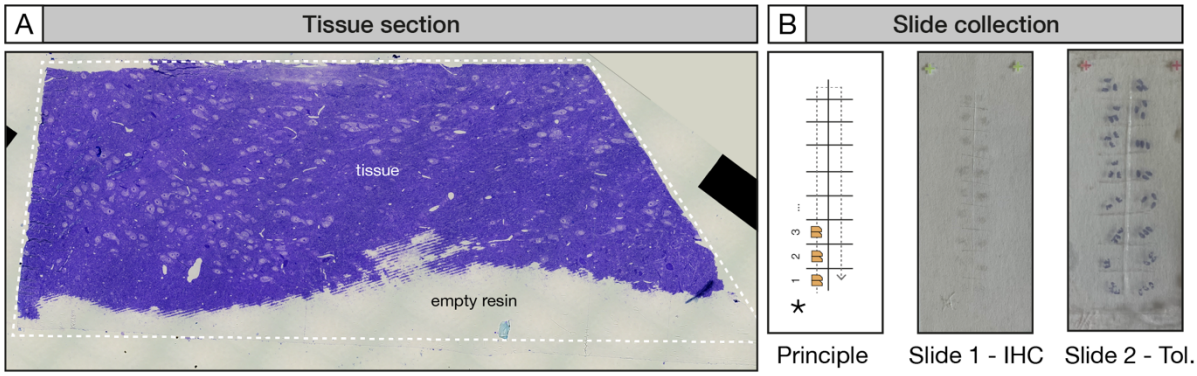

Figure 10. CLEM sectioning. A) Toluidine blue staining can be performed on-the-fly to assess the proportion of tissue/resin on the cut sections; B) Schematic showing the markings on the underside of the glass slide etched with a diamond scribe. The grid delineates where the sections should be placed for each collection cycle and a mark (*) denoting the start position. The numbers indicate the order of collection. Sections can be collected sequentially on multiple glass slides within each CLEM cycle to be used for multiple antibodies in IHC and / or toluidine blue staining. Example images of unstained sections (Slide 1-IHC) and toluidine blue stained sections (Slide 2-Tol.) placed on the glass slides are shown.

Immunohistochemistry (IHC) (Figure 11)

Timing: 1 day

CRITICAL- Through the whole protocol don't let the slides dry. If many slides are stained at the same time, process them in batches. Be careful not to scratch the surface of the section during the manual handling of the slides throughout this section.

Preparation

Timing: 12 hours

73. At least 12 hours before preparing the etching solution in a glass staining jar.

Etching

Timing: 5 minutes

74. Place the glass slides into the glass staining jar containing the etching solution and etch for 3 minutes.
    NOTE- Use a dedicated waste for disposal of the etching solution.
75. Rinse glass slides in 100% Ethanol.

(OPTIONAL) Pre-treatment/antigen retrieval

Timing: 45 minutes

76. Rinse slides in ddH2O.
77. Put glass slides in 100% Formic acid for 10 min.
    NOTE- Work under the fume-hood.
78. Rinse slides in ddH2O.
79. Put glass slides in 1 x Tris-EDTA, pH 9 or 1 x NA-Citrate, pH 6 (depending on the antibody) for 30 minutes at 100°C.
    NOTE- Use a plastic staining jar with a lid to avoid evaporation and place it in the steamer.
80. Wash slides in ddH2O to cool down the slides.

Blocking

Timing: 30 minutes

81. Put slides in the endogenous HRP blocking solution for 10 minutes at RT.
82. Wash slides in PBS 3 times (quick wash, quick wash, 5 minutes)

PAUSE POINT- Slides can stay in PBS for a few days.

83. Place all the slides on the staining tray and use a pathology pen to draw an area around the sections on the glass slide to contain the staining. NOTE- Put some water in the bottom of the staining tray to keep the environment moist.
84. Add a few drops of antibody diluent inside the area and leave it for 10 minutes.

Antibody staining

Timing: 2 hours - overnight depending on antibody

85. Prepare the correct dilution of the primary antibody in the antibody diluent.

CRITICAL- Primary antibody concentration, timing, incubation temperature, and antigen retrieval are highly dependent on the specific antibody and needs to be optimized prior. Common time and temperature combinations are 1 hour at 37°C, 4 hours at RT, overnight at 4°C.

86. Incubate the primary antibody according to its working time/temperature combination.

CRITICAL- Prepare PBS-T solution during primary antibody incubation.

87. Wash slides 3 times in PBS-T (quick rinse, quick rinse, 5 minutes incubation)
88. Dry the pathology pen marks on the glass slides by rolling the end of a cotton tip over the surface of the pen mark.
    NOTE- The detergent stops the pathology pen creating a liquid boundary. Avoid touching the area where the sections are.
89. Add a few drops of secondary antibody on glass slides and incubate for 30 minutes at RT.
    NOTE- Make sure to match primary and secondary antibodies species.

90. Wash slides 3 times in PBS (quick rinse, quick rinse, 5 minutes incubation).

PAUSE POINT- Slides can stay in PBS for a few days.

91. Prepare HRP-chromogen detection solution.
92. Stain slides with HRP-chromogen solution for 3 minutes at RT.
93. Rinse slides in 1 x PBS.
94. Place slides in ddH2O to stop the reaction.
95. Contrast the slides with hematoxylin staining (protocol in Supplementary information)
96. Use a mounting medium to coverslip the slides.
97. Leave the slides in the oven for 1 hour at 37°C or overnight at RT to let the mounting medium dry.

PAUSE POINT- The slides can be dried at this point by inverting and gently pressing against some absorbent material. Dried slides can be kept for a few weeks without any changes.

(OPTIONAL) Toluidine Blue staining

Timing: 1-12 hours

Counterstaining a glass slide with toluidine blue can help with the tissue morphology identification and for sample correlation/alignment purposes.

98. Cover the sample on the glass slide with a droplet of toluidine blue solution.
99. Heat the slide on the hot plate (at roughly 100°C) for about 30 seconds, or until the droplet edge dries (turns green).
100. Rinse with ddH2O until the droplet edge has been cleared.
101. Dry the slide on the hot plate for a few seconds until the water evaporates.
102. Repeat steps 96 and 97 to coverslip the slides.

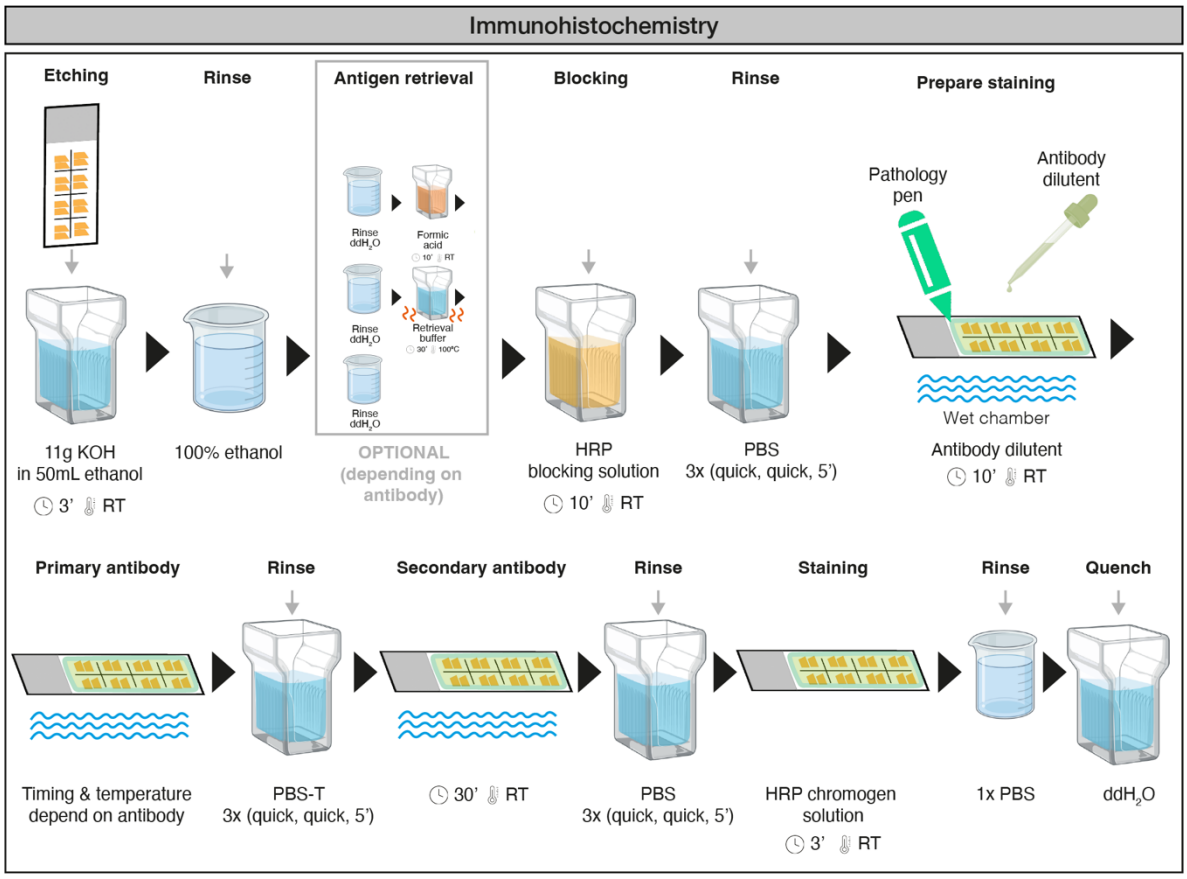

Figure 11. Schematic summary outlining IHC staining of glass slides.

Imaging of stained glass slides

Timing: 2 hours per sample

103. Image the glass slides processed for IHC and toluidine blue with a light microscope at the magnification necessary to see the tissue features and location of stained ROI.
104. Annotate cycles containing ROIs showing positive IHC staining. They will be used to find the corresponding EM grids in the next step.
105. The IHC and toluidine blue images for each cycle can be aligned here using FIJI bigwarp to follow the ROI through the block (steps 106-115).

Simple Image correlation using BigWarp

Timing: 5 minutes

Here we can align IHC or toluidine blue images to EM images.

106. Open FIJI.
107. Load the images to be aligned

NOTE- A rough alignment can first be performed by flipping and/or rotating the images. To do so, click Image >Transform.

108. Click Plugins > BigDataViewer > Big Warp.
109. Define which image will be the moving and target image. For example:
    a. IHC (moving) onto toluidine blue (target)
    b. IF (moving) onto toluidine blue (target)
110. Press the spacebar to toggle the landmark mode on/off.
111. Click a point on one image and click the same point on the other image to add the landmarks. Add at least 4 landmarks.
112. (OPTIONAL) File > save landmarks.
113. Press T to transform the moving image.
114. Click File > Export moving Image.
115. Save the resulting image.

NOTE- Check the image alignment by making the images into stacks. Click Image > Stack > Images to Stack.

Fluorescence Image correlation using BigWarp

Timing: 2 hours

Correlation of a fluorescence volume taken on a free-floating section presents a challenge when aligning with an ultrathin 200 nm LM image or 80 nm TEM images. The fluorescent and LM/TEM images are often deformed in respect to each other in a non-linear way and some additional correlation steps are needed.

116. Make a maximum projection of the fluorescence volume showing the DAPI and Brightfield channel.

NOTE- The brightfield channel is useful to provide tissue context to help orient the fluorescence sample with the toluidine blue/ IHC /EM images. Other

channels/physical fiducial markers which would help with the orientation can be used instead/additionally.

117. Take a snapshot of the image with high enough resolution to resolve individual cell nuclei.
118. Align all the toluidine blue/IHC images acquired from all the CLEM cycles collected from the block volume (steps 69).
    a. Align the image from cycle 1 (target) to the image from cycle 2 (moving).
    b. Transform and save the moving image (cycle 2 transform).
    c. Align the cycle 2 transform (target) to the image from cycle 3 (moving).
    d. Transform and save the moving image (cycle 3 transform).
    e. Continue until all the images have been transformed onto the image from the previous cycle.
119. Close all non-transformed images (except for cycle 1).
120. Click Image > Stack > Images to Stack.
121. Scroll through the stack to check the alignment and visualize the features as they move through the volume.
122. Align the fluorescence to the toluidine blue stack.
    a. Separately open one image in the stack to serve as the toluidine reference image.
    b. Open the fluorescence maximum projection.
    c. Perform a basic alignment of the fluorescence (moving) and toluidine blue (target) using any features in the tissue that can be aligned confidently. (steps 106-115).
    d. Press T to transform the fluorescent image.
    e. Save the fluorescent transform.
    f. Add the fluorescence transform to the aligned toluidine blue stack adjacent to the position of the reference toluidine blue image.
    g. Move back and forth between the images to identify nuclei or other tissue features that correlate between the fluorescent transform and the reference toluidine blue images.
    h. Scroll up and down the stack to identify features or cell nuclei that become visible in different planes within the stack.

i. Add the newly correlated nuclei/features as additional landmarks in BigWarp.
j. Repeat steps 122 d-i until the region surrounding the ROI has been correlated with a high level of confidence.
k. Save the landmarks.

NOTE- The same transformation can be applied to different images. For example, if you first warp the fluorescence to toluidine blue (T1) and toluidine blue to EM (T2), you can directly warp the fluorescence to the EM by doing a T1>T2 transformation. In that case, you can only re-use the T2 transformation matrix if the pixel size of all images are set correctly, and the coordinates of the landmarks are saved in a unit that makes sense (so µm and not pixels). The pixel size can be adjusted in FIJI through the menu: Image > Properties. Use the same units for all images (e.g., µm).

EM image acquisition

123. Find the EM grid corresponding to the cycle containing the positive IHC staining on the glass slide or recognized in the toluidine blue staining.
124. Load the EM grid in a TEM.
125. Use easy recognizable features in the tissue (e.g. cell nuclei, blood vessels, resin section edges) to locate the ROI (s) on the grid corresponding to the positive IHC signal.
126. (OPTIONAL) CLEM4CINA can be used to help locate the ROI on the grid (Supplementary Information; repository link https://github.com/LBEM-CH/clem4cina).
127. Acquire EM images / tomography as desired.
128. Correlate with FIJI BigWarp again as needed.

Timing

The timing does not include the reagent preparation.

Brain Fixation, Immunolabeling and fluorescent imaging of the sections

Steps 1-15: 24 hrs on 1 cm^3 brain blocks. 2 h on vibratome sections

Steps 16-23: 2.5 hrs plus overnight incubation

Steps 24-31: 1-3 hrs per brain slice, depending on number z-stacks taken

En bloc resin embedding, laser capture microdissection and serial sectioning of resin blocks

Steps 32-52: Resin embedding (full protocol) 2.5 days plus 48 hrs curing time; Resin embedding (short protocol) 1 day plus 48 hrs curing time

Steps 53-56: 1 h per brain slice

Steps 57-72: 2 hrs per resin block

Immunohistochemistry and Imaging of stained glass slides

Steps 73-96: < 1 d, depending on time needed for antibody incubation (1 h, 3 hrs, overnight)

Steps 97-102: 1-12 hrs depending on the temperature used for the mounting medium

Steps 103-105: > 2 hrs per sample

Image correlation and EM image acquisition

Steps 106-122: > 2 hrs, depending on number of samples needing correlation

Steps 123-128: > 1 d, depending on the number of images needed

Troubleshooting

Step	Problem	Possible Reason	Solution
25	There is no fluorescent signal for the antibody	a) The antibody penetration is poor. b) the antibody penetration depth is limited c) the antibody requires antigen retrieval.	a) longer antibody incubation times are required. b) antibody staining should be performed on thinner vibratome sections c) Antigen retrieval may be required. We have tested limited heat-mediated antigen retrieval methods on post-mortem human brain tissue without significant deterioration of the global ultrastructure (Supplementary Figure 8). However, this should be extensively tested for each case to assess the impact on the

			ultrastructural details of interest.
26	Fluorescence signal and resolution are too low to accurately map the ROIs.	The overview has been taken in too low a magnification, or too small an area within a larger tissue section.	Take a brightfield image to capture large morphological details in the tissue or take a montage at higher magnification and stitch the images together.
43	Lead aspartate precipitates.	NaOH isn't fresh or the solution isn't at 60°C or pH is above 5.5.	Prepare fresh 1M NaOH solution and keep all solutions in a 60°C water bath during the pH adjustment and Ensure the pH is adjusted only with NaOH and not corrected with acid if the pH goes above pH 5.5
53-b-ii	The image alignment is complicated due to the tissue distortion or breakage during the embedding process.	The resin embedded sections are brittle or the resin embedding process causes shrinkage of the sections.	Only parts of the tissue that are closest to the ROIs need to be aligned precisely enough to draw the positions of the block to be excised. The BigWarp plugin in FIJI can be used to create the overlays with high precision (steps 106-115).
53-b-v	The resin embedded shape has not been cut completely by the laser.	The resin on that part of the section is thicker than for other regions.	Redo the laser cutting on the same region until it is completely cut.
53-b-vi	It is difficult to distinguish the blocks from each other on the tape after their excision from the section.	There are too many blocks with the same shape and/or they are in a different orientation to the empty regions left in the sections.	Make distinctive shapes for the different blocks, cut identifying features within the block, and/or use a binocular to pick them up from tape.
53-b-vi	The section does not stick to the tape during excision of the laser cut blocks.	The ROI has not been cut completely by the laser.	Use tweezers to remove the section.
83	There is a film over the sections that is not stained by the antibody, toluidine blue or hematoxylin.	The pathology pen was not completely dry before the antibody diluent was added to the slide causing a thin film of pen to form on top of the liquid surface. The film remains on the slide covering the sections when the liquid is tipped off preventing any further staining of the	Ensure the pathology pen is completely air-dried before adding the antibody diluent to the slide.

		section	
89	The secondary antibody leaks all over the slide and isn't contained within the pathology pen.	The detergent from the PBS-T was not sufficiently removed from the surface of the slide.	Dry the surface of the pathology pen by rolling the end of a cotton bud over the pen boundary. Take care not to touch the sections.
96	Bubbles have formed between coverslip and the glass slide.	The air bubbles were formed when the coverslip was placed on the mounting medium	Before the glue dries, gently press the coverslip on the glass slide with the help of a pipette tip to remove the air bubbles.
104	No positive immunostaining was observed on the glass slides.	a) The staining conditions for the antibody need optimizing. b) the distribution of the epitope is too diffuse to be observed in the ultra-thin sections	a) Test different antibody concentrations, incubation times and temperatures and common antigen retrieval methods. b) cut thicker sections for the glass slides (e.g. 500 nm) to increase the number of epitopes available for staining in the sections.
122	Multiple features can not be correlated between fluorescence and LM/EM.	The section was not completely flat during either the fluorescent imaging of the free-floating section, or the resin embedding causing large empty regions of empty resin within the ultra-thin sections impeding successful correlation, and/or the features that are visible on the individual ultrathin sections to be in different z-planes compared to what is anticipated based on the individual steps within the fluorescent z-stack.	Ensure the free-floating section is completely flat under the coverslip before fluorescent imaging. Cut thicker vibratome sections to aid in the flat-embedding.
127	Poor ultrastructure by EM.	The sample has not been properly fixed before resin embedding, or the sample has not been properly infiltrated during resin embedding	a) If using disease models, compare the ultrastructure to that of a control (non-diseased) sample prepared in the same way to ensure the ultrastructure is not disease-related.

			b) Increase the amount of glutaraldehyde used in the initial fixation solutions (up to 2.5%). c) Ensure there has been no errors in the resin-embedding process, that all steps were carefully followed and the samples were not allowed to dry out between steps. d) Increase infiltration times for the resin embedding protocol used.
128	Bad correlation between IHC/toluidine blue and EM.	The field-of-view of the EM micrograph is too limited for a good correlation.	We recommend taking two micrographs of each structure of interest: (1) an overview micrograph, i.e. a large FOV at low nominal magnification (typically around 630x) and (2) a detailed micrograph, i.e. a small FOV at high nominal magnification (typically 8400x). Step 3 can then be split in two substeps, namely. 3a. Toluidine blue (moving) to overview micrograph (target). 3b. Overview micrograph (moving) to detailed micrograph (target).

Anticipated results

Using this protocol, it should be possible to perform CLEM on post-mortem human brain tissue as well as other biological samples (Figure 12) resulting in the correlation of fluorescence, brightfield and EM images. The example below has been collected from the brain of a patient suffering from multiple systems atrophy with 4 hours post-mortem delay and fixed at autopsy in the fixation buffer for 75 hours. The fixed brain block was cut into 40 µm vibratome sections and immunostained with a neurofilament antibody cocktail to detect the neurons (neurofilament H, abcam ab5539; MAP2, abcam ab5392) and DAPI to detect cell nuclei (BioLegend cat # 422801). Following the creation of a fluorescent map, the sample was processed for electron microscopy, serial sectioning and electron microscopy image

acquisition. The sections on glass slides were processed for IHC against alpha synuclein (pS129, abcam ab51253) and the subsequent alignment of fluorescence images, brightfield images (toluidine blue and IHC) and EM images enables us to correlate a single cell observed by fluorescence microscopy with its EM ultrastructure. Here, the correlation for a single neuron (n) has been done using four nearby nuclei (N) in the surrounding tissue. Combining a short post-mortem delay, optimized fixation solutions and correlation techniques enables us to achieve excellent preservation of ultrastructure (lipidic organelles) of the post-mortem human brain tissue and optimal precision in defining ROIs. and from RT-CLEM and opening up new insights of the mechanisms behind the neurodegenerative diseases. This protocol should give consistent results in terms of ultrastructural quality for a given sample, if the sample was properly fixed and stored. Membranes, organelles, etc. should be well preserved in tissue using our inclusion criteria described previously.

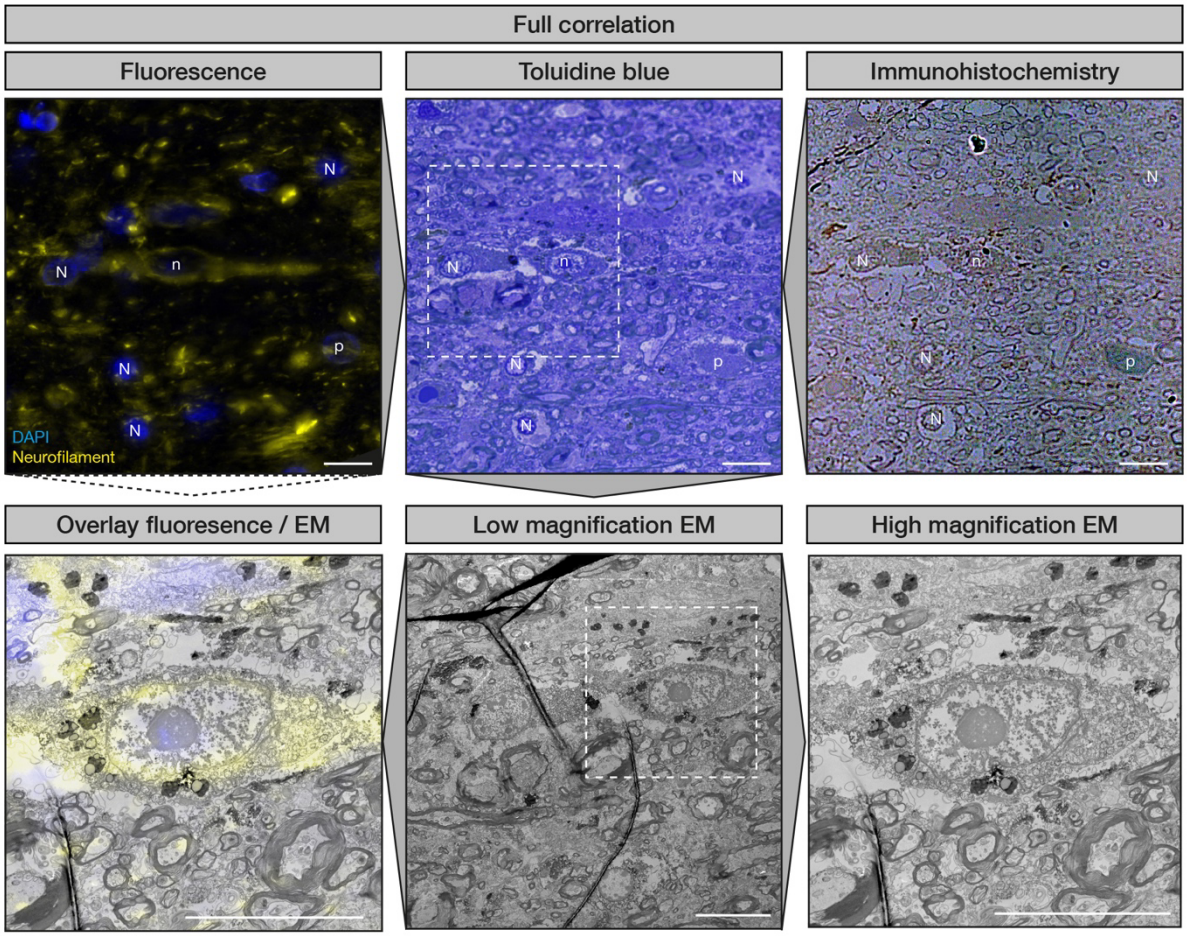

Figure 12. The full CLEM pipeline shown for the fluorescence and EM correlation of a single neuron within post-mortem human brain tissue. Fluorescent map generation and correlation to toluidine blue and IHC images in preparation for EM overlay. For correlation the same nuclei (N) are used that are visible in fluorescence, toluidine blue, and IHC. The target nucleus of the neuron desired for imaging is depicted with n. Typically, with

IHC correlation immunostained regions of interest can be identified, as indicated by (p). (NB: not all nuclei observed in the fluorescence volume are visible in the single-plane LM images). Toluidine blue images are then correlated to low magnification EM images to identify the correct cells in EM. Based on the correlation of fluorescence with toluidine blue/IHC images and the correlation between toluidine blue/IHC images with EM images, fluorescence images can be overlaid with EM images with precision. High magnification fluorescent z-stacks were deconvoluted using the THUNDER algorithm (Leica). Scale bar is 10 µm.

Author contributions

AJL, SHS, CG, WvdB and HS conceived the strategy for carrying out CLEM on post-mortem human brain samples. WvdB collected, dissected and processed the brain tissue samples for CLEM. AJL and WvdB developed the strategy for fluorescent staining, imaging and mapping of free-floating brain sections with further optimisation by DS, NS, and DB. AJL optimized the immunohistochemistry of resin sections on glass slides with contributions from MDF for the toluidine blue staining. CG optimized the manual *en bloc* staining and alternate serial sectioning protocol for 60 µm human brain sections. JD designed, printed and tested the sample holder components, and optimized the embedding protocol for the tissue auto-processor. LvdH wrote the macro to create .xml files. AJL and MDF optimized cutting of resin samples by laser-capture microdissection. DB and LvdH optimized the strategy for the image correlation of fluorescence and light microscopy with electron microscopy. CB and HS wrote the CLEM4CINA program. AJL tested the protocol on mouse brain sections and cell monolayers. NS, DS, MDF, DB, LvdH and AJL wrote the manuscript and prepared the figures. All authors approved the final version of the manuscript.

Acknowledgments

We would like to thank the Netherlands brain bank autopsy team and Dr Jürgen Hench at the University Hospital Basel for the collection of post-mortem human brain samples used for the development of this protocol, the EPFL/UNIL workshop for printing the autoprocessor components, the Leica Research & Development team for assistance in the optimization of cutting resin samples with the Laser capture microdissection instrument, the staff at the Electron Microscopy Facility (UNIL) for training on equipment and TEMs and Dr. Ricardo Righetto (University of Basel) for assistance with the CLEM4CINA program. AJL was

supported by the Synapsis Foundation Switzerland (Grant no. 2019-CDA01) and Parkinson Schweiz. This work was in part supported by the Swiss National Science Foundation (SNF Grants CRSII5_177195 and 310030_188548). WvdB is supported by the Dutch Parkinson association (Grant no. 2020-G01). Figure 1, Figure 11 and Supplementary Figure 6 were created with BioRender.com with agreement numbers MY26TF0GE3, BF26TAAJ8K and GQ26TAAMU1 respectively.

Competing interests

The authors declare no competing interests.

Policy considerations

All brain samples used for this study were collected from patients who participated in the Netherlands Brain Bank donation program (www.brainbank.nl). All donors provided written informed consent for a brain autopsy and the use of the material and clinical information for research purposes. Detailed neuropathological and clinical information was made available, in compliance with local ethical and legal guidelines, and all protocols were approved by Vrije University Medical Center institutional review board. Demographic features and clinical symptoms were abstracted from the clinical files, including sex, age at symptom onset, age at death, disease duration, presence of dementia, core and supportive clinical features for the diseases.

Data Availability

Images shown in this manuscript can be made available upon request.

Code Availability

All codes are available on github. CLEM4CINA.py can be accessed through the public repository with access under: https://github.com/LBEM-CH/clem4cina. The FIJI macro can be accessed through the public repository with access under: https://github.com/LBEM-CH/clem-for-human-brain.

References

1. Hein, B., Willig, K. I. & Hell, S. W. Stimulated emission depletion (STED) nanoscopy of a fluorescent protein-labeled organelle inside a living cell. *Proc Natl Acad Sci U S A* **105**, 14271–14276 (2008).

2. Lau, L., Lee, Y. L., Sahl, S. J., Stearns, T. & Moerner, W. E. STED Microscopy with Optimized Labeling Density Reveals 9-Fold Arrangement of a Centriole Protein. *Biophys J* **102**, 2926–2935 (2012).
3. Schermelleh, L. *et al.* Super-resolution microscopy demystified. *Nat Cell Biol* **21**, 72–84 (2019).
4. Sarkar, D. *et al.* Revealing nanostructures in brain tissue via protein decrowding by iterative expansion microscopy. *Nat Biomed Eng* **6**, 1057–1073 (2022).
5. Morgan, C., Godman, G. C., Breitenfeld, P. M. & Rose, H. M. A CORRELATIVE STUDY BY ELECTRON AND LIGHT MICROSCOPY OF THE DEVELOPMENT OF TYPE 5 ADENOVIRUS : I. ELECTRON MICROSCOPY. *Journal of Experimental Medicine* **112**, 373–382 (1960).
6. Godman, G. C., Morgan, C., Breitenfeld, P. M. & Rose, H. M. A CORRELATIVE STUDY BY ELECTRON AND LIGHT MICROSCOPY OF THE DEVELOPMENT OF TYPE 5 ADENOVIRUS : II. LIGHT MICROSCOPY. *Journal of Experimental Medicine* **112**, 383–402 (1960).
7. Knott, G. W., Holtmaat, A., Wilbrecht, L., Welker, E. & Svoboda, K. Spine growth precedes synapse formation in the adult neocortex in vivo. *Nat Neurosci* **9**, 1117–1124 (2006).
8. Trachtenberg, J. T. *et al.* Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* **420**, 788–794 (2002).
9. Shahmoradian, S. H. *et al.* Lewy pathology in Parkinson's disease consists of crowded organelles and lipid membranes. *Nature Neuroscience* **22**, 1099–1109 (2019).
10. Böing, C. *et al.* Distinct ultrastructural phenotypes of glial and neuronal alpha-synuclein inclusions in multiple system atrophy. *Brain* awae137 (2024) doi:10.1093/brain/awae137.
11. De Rossi, P. *et al.* FTLD-TDP assemblies seed neoaggregates with subtype-specific features via a prion-like cascade. *EMBO Rep* **22**, e53877 (2021).
12. Tatsumi, S. *et al.* Ultrastructural differences in pretangles between Alzheimer disease and corticobasal degeneration revealed by comparative light and electron microscopy. *Acta Neuropathol Commun* **2**, 161 (2014).
13. Tanner, H., Sherwin, O. & Verkade, P. Labelling strategies for correlative light electron microscopy. *Microscopy Research and Technique* **86**, 901–910 (2023).
14. de Boer, P., Hoogenboom, J. P. & Giepmans, B. N. G. Correlated light and electron microscopy: ultrastructure lights up! *Nat Methods* **12**, 503–513 (2015).
15. Müller, A. et al. Modular segmentation, spatial analysis and visualization of volume electron microscopy datasets. *Nat Protoc* **19**, 1436–1466 (2024).
16. Lam, I. *et al.* Rapid iPSC inclusionopathy models shed light on formation, consequence and molecular subtype of α-synuclein inclusions. *bioRxiv* 2022.11.08.515615 (2022) doi:10.1101/2022.11.08.515615.
17. Lewis, A. J. *et al.* Imaging of post-mortem human brain tissue using electron and X-ray microscopy. *Current Opinion in Structural Biology* **58**, 138–148 (2019).
18. Karreman, M. A. *et al.* Fast and precise targeting of single tumor cells in vivo by multimodal correlative microscopy. *J Cell Sci* **129**, 444–456 (2016).
19. Collinson, L. M., Carroll, E. C. & Hoogenboom, J. P. Correlating 3D light to 3D electron microscopy for systems biology. *Current Opinion in Biomedical Engineering* **3**, 49–55 (2017).
20. Lane, R., Wolters, A. H. G., Giepmans, B. N. G. & Hoogenboom, J. P. Integrated Array Tomography for 3D Correlative Light and Electron Microscopy. *Front Mol Biosci* **8**, 822232 (2022).
21. Kolotuev, I. Work smart, not hard: How array tomography can help increase the ultrastructure data output. *J Microsc* (2023) doi:10.1111/jmi.13217.
22. Micheva, K. D. & Smith, S. J. Array tomography. *Neuron* **55**, 25–36 (2007).
23. Kuzuhara, S., Mori, H., Izumiyama, N., Yoshimura, M. & Ihara, Y. Lewy bodies are ubiquitinated: A light and electron microscopic immunocytochemical study. *Acta Neuropathol* **75**, 345–353 (1988).
24. Allsopp, G. & Gamble, H. J. Light and electron microscopic observations on the development of the blood vascular system of the human brain. *J Anat* **128**, 461–477 (1979).
25. Castejon, O. J., Castejon, H. V., Diaz, M. & Castellano, A. Consecutive light microscopy, scanning-transmission electron microscopy and transmission electron microscopy of traumatic human brain

oedema and ischaemic brain damage. *Histol Histopathol* **16**, 1117–1134 (2001).
26. de Boer, P., Hoogenboom, J. P. & Giepmans, B. N. G. Correlated light and electron microscopy: ultrastructure lights up! *Nat Methods* **12**, 503–513 (2015).
27. Genoud, C., Titze, B., Graff-Meyer, A. & Friedrich, R. W. Fast Homogeneous En Bloc Staining of Large Tissue Samples for Volume Electron Microscopy. *Front. Neuroanat.* **12**, (2018).
28. Hua, Y., Laserstein, P. & Helmstaedter, M. Large-volume *en bloc* staining for electron microscopy-based connectomics. *Nat Commun* **6**, 7923 (2015).
29. Deerinck, T. J., Bushong, E. A., Ellisman, M. H. & Thor, A. Preparation of Biological Tissues for Serial Block Face Scanning Electron Microscopy (SBEM). (2022).
30. Kopek, B. G. *et al.* Diverse protocols for correlative super-resolution fluorescence imaging and electron microscopy of chemically fixed samples. *Nat Protoc* **12**, 916–946 (2017).
31. Maco, B. *et al.* Semiautomated correlative 3D electron microscopy of in vivo-imaged axons and dendrites. *Nat Protoc* **9**, 1354–1366 (2014).
32. Liu, M. *et al.* Tracking endocytosis and intracellular distribution of spherical nucleic acids with correlative single-cell imaging. *Nat Protoc* **16**, 383–404 (2021).
33. Darcy, K. J., Staras, K., Collinson, L. M. & Goda, Y. An ultrastructural readout of fluorescence recovery after photobleaching using correlative light and electron microscopy. *Nat Protoc* **1**, 988–994 (2006).
34. Kay, K. R. *et al.* Studying synapses in human brain with array tomography and electron microscopy. *Nat Protoc* **8**, 1366–1380 (2013).
35. Madeleine A. G. Gilbert *et al.* In situ cryo-electron tomography of β-amyloid and tau in post-mortem Alzheimer's disease brain. *bioRxiv* 2023.07.17.549278 (2023) doi:10.1101/2023.07.17.549278.
36. Wagner, F. R. *et al.* Preparing samples from whole cells using focused-ion-beam milling for cryo-electron tomography. *Nat Protoc* **15**, 2041–2070 (2020).
37. Schaffer, M. *et al.* A cryo-FIB lift-out technique enables molecular-resolution cryo-ET within native Caenorhabditis elegans tissue. *Nat Methods* **16**, 757–762 (2019).
38. August, B. K., Kong, L., Massey, R. J., Zhang, S.-C. & Strader, T. E. Robotic Optimization of Specimen Preparation Protocol for Astrocytes Seeded on Coverslips for Imaging by Transmission Electron Microscopy (TEM). *Microscopy and Microanalysis* **29**, 1106–1110 (2023).
39. Mäntylä, E. & Verkade, P. Some Guiding Principles for a "Simple" Correlative Light Electron Microscopy Experiment. in *Imaging Cell Signaling* (eds. Wuelfing, C. & Murphy, R. F.) 89–102 (Springer US, New York, NY, 2024). doi:10.1007/978-1-0716-3834-7_8.
40. Bogovic, J. A., Hanslovsky, P., Wong, A. & Saalfeld, S. Robust registration of calcium images by learned contrast synthesis. in *2016 IEEE 13th International Symposium on Biomedical Imaging (ISBI)* 1123–1126 (2016). doi:10.1109/ISBI.2016.7493463.
41. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676–682 (2012).
42. Navarro, P. P. *et al.* Cerebral Corpora amylacea are dense membranous labyrinths containing structurally preserved cell organelles. *Scientific Reports* **8**, 18046 (2018).
43. Tamada, H., Blanc, J., Korogod, N., Petersen, C. C. & Knott, G. W. Ultrastructural comparison of dendritic spine morphology preserved with cryo and chemical fixation. *Elife* **9**, e56384 (2020).
44. Korogod, N., Petersen, C. C. & Knott, G. W. Ultrastructural analysis of adult mouse neocortex comparing aldehyde perfusion with cryo fixation. *eLife* **4**, e05793 (2015).
45. Griffiths, G. *Fine Structure Immunocytochemistry*. (Springer Science & Business Media, 2012).
46. Mollenhauer, H. H. Artifacts caused by dehydration and epoxy embedding in transmission electron microscopy. *Microscopy Research and Technique* **26**, 496–512 (1993).
47. Schnell, U., Dijk, F., Sjollema, K. A. & Giepmans, B. N. G. Immunolabeling artifacts and the need for live-cell imaging. *Nat Methods* **9**, 152–158 (2012).