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🌐 Protocol for Electron Microscopy Analysis of Internal Limiting Membrane and Epiretinal Membrane from Vitrectomy Surgery

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We use this protocol and it's working

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Abstract

The epiretinal membrane (ERM), a fibrocellular layer on the retinal surface, significantly impacts visual function. This protocol outlines a cross-sectional study designed to investigate the pathophysiology of ERM formation by comparing internal limiting membrane (ILM) pathology in patients with and without ERM. ILM and ERM specimens will be collected during pars plana vitrectomy and analyzed using light and transmission electron microscopy to assess morphological and ultrastructural changes.

We expect to identify distinct pathological differences in ILMs associated with ERM, including variations in cellular infiltration and membrane structure. These findings may support the hypothesis that ILM alterations contribute to ERM development; alternatively, the absence of such differences would suggest involvement of other pathogenic pathways.

Guidelines

Prior to any study procedures, participants must provide written, informed consent. We adhered to the principles outlined in the Declaration of Helsinki. All authors reported no conflict of interest to declare.

Materials

1. Usual pars plans vitrectomy tools
2. Cryovial tubes (1.8mL size recommended) each prefilled with 3% glutaraldehyde in 0.1M phosphate buffer at pH 7.3
3. Sterile filter paper
4. Temperature-controlled refrigerator at 4°C for 3% glutaraldehyde in 0.1M phosphate buffer at pH 7.3 storage
5. Phosphate buffer
6. 4% osmium dioxide
7. Gelatin
8. Ethanol
9. Resin
10. Ultramicrotome cutter
11. Light microscope
12. Transmission electron microscope

Safety warnings

 Please note that specimen loss is possible, varying between the specimen handling to processing.

Ethics statement

This protocol and informed consent forms have been approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (COA No. 0948/2023).

Before start

This protocol was developed to investigate the role of the internal limiting membrane (ILM) in the pathogenesis of epiretinal membrane (ERM) formation. Prior to initiating the study, ethical approval was obtained, and standardized surgical and specimen processing workflows were established. All participating surgeons and research staff were trained to ensure consistency in specimen collection, handling, and documentation. This protocol is intended to guide a structured comparison of ILM pathology in patients with and without ERM, using both light and transmission electron microscopy to explore morphological and cellular differences.



Preparation

- 1 Prepare 1.8 mL cryovial tubes at the operating theatre.
- 2 Prepare a sterile filter paper to ease the specimen collection process

Specimen Harvesting

1h 22m

- 3 Each participant will undergo their surgical procedures as usual, which will be either a 25G or 27G PPV with ILM and/or ERM peeling. 1h
- 4 During the membrane peeling process, the surgeon will stain the membrane using brilliant blue G until it is visualized for peeling. 2m
- 5 Subsequently, the surgeon will perform continuous curvilinear membrane peeling using forceps, targeting either the ILM alone or both the ILM and ERM, depending on intraoperative findings. 10m
- 5.1 In cases where the ILM and ERM are present together, we aim to collect both membranes for histopathological examination. If the ILM and ERM are separated, multiple peels may be required, and all tissue will be collected into the same specimen tube.
- 5.2 In cases where the ILM and ERM are inseparable, the membranes may be removed in one or more passes at the surgeon's discretion to obtain the maximum amount of tissue for processing.
- 6 All specimens will be extracted from the intraocular space via the vitrectomy port using forceps. 5m
- 7 Afterward, the specimens will be placed on sterile filter paper, folded, and fixed in the tube. 5m

Specimen Processing

1h 40m

- 8 Each tube will undergo a phosphate buffer washout to replace the 3% glutaraldehyde with 0.1M phosphate buffer at pH 7.3 20m
- 9 The specimen will be fixed in 4% osmium dioxide, causing it to change color to a dark brown hue, and then embedded in gelatin, with the filter paper removed. 20m



9.1 If the specimen is not visible at this stage, the process will be halted, and it will be marked as specimen loss during delivery.

10 If the specimen is visible, it will undergo dehydration in graded ethanol, followed by embedding in resin and processing using an ultramicrotome cutter to obtain the specimen layer for LM examination.

1h

Light microscopy examination

35m

11 The pathologist will then examine the specimen using LM.

11.1 Pathologist will assess each specimen and determine its presence or absence. If present, the specimen's colorization will be described as either adequate or inadequate.

5m

11.2 If the specimen is absent at this stage, the EM examination will be discontinued, and it will be marked as specimen loss during processing.

5m

11.3 If the specimen is present, it will later be examined using the JEOL-JEM 1400 plus transmission electron microscope (TEM).

12 Pathologist will then examine cell counting, conducted at a high-power field (400x), with up to 10 fields examined (1-10 depending on specimen size). The cell count will be calculated as cells per 1 mm².

20m

13 Additional descriptions will be provided for any significant findings observed during the examination.

5m

Transmission electron microscopy examination

3h 25m

14 In the JEOL-JEM 1400 plus TEM, the scan results were marked by a grid. We selected the grid with the highest specimen-to-grid ratio.

5m

15 **Internal limiting membrane** examination

40m

15.1 400x Whole grid examination - marking maximum of 10 boxes for ultrastructure examination (depends on specimen sizes). Zooming in can be done to confirm each box has the ILM within the grid.

20m

15.2	8000x Vacuolization examination – examining for vacuolization of all selected boxes. Zooming in can be done to assess the appearance of the vacuoles.	10m
15.3	8000x ILM surface description - both retinal side and vitreous side	5m
15.4	20000x ILM appearance description – such as degree of looseness.	5m
16	Epiretinal membrane examination	1h
16.1	400x Whole grid examination – marking the presence of the ERM and select the grid with highest cellularity for the description of the cell types. The characteristics of each cell type were as mentioned by Smiddy et al.(16), Gandorfer et al.(17), Beyazyildiz et al.(18), and Frisina et al.(21)	40m
16.2	20000x Content description – such as native collagen fibers and newly formed collagen. The appearance of the collagen fibers was as mentioned by Smiddy et al.(16), Gandorfer et al.(17), Beyazyildiz et al.(18), Kritzenberger et al.(19), and Regoli et al.(20)	20m

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