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Use of electron microscopy in disease diagnosis of finfishes and shellfishes

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Understanding the pathogen and the pathogenesis at cellular levels are imperative in the studies of disease causing organisms. With its very high resolving and magnifying powers, Electron Microscopy has opened up new vistas in studying the ultra structure and has become an indispensable tool in understanding many of the diseases and their etiological agents. Transmission electron microscopy (TEM) can reveal the ultra structural details at cellular levels, whereas Scanning electron microscopy (SEM) can show the morphology of minute structures/organisms in three-dimensional state. Combining the TEM and SEM, it has become possible to study and classify the viruses and virus-like organisms. Among the spectrum of diagnostic techniques, electron microscopy remains the most important tool to establish a viral etiology in the case of disease outbreaks without any previous history, and stands out as the only technique, which can visualize and record viral pathogenesis at cellular levels.

1. The Instrument, principle and utilities

Electron Microscopes are instruments that use a beam of highly energetic electrons to examine objects on a very fine scale. The limitations of Light Microscopes, low magnifying and resolving powers (1000 x magnification and a resolution of 0.2 micrometers) paved the way for the development of electron microscopes. Transmission Electron Microscope (TEM) was first developed by Max Knoll and Ernst Ruska in Germany in 1931. Scanning Electron Microscope (SEM) was developed in 1942. Electron Microscopes function exactly like their optical counterparts, except that they use a focused beam of electrons instead of light to "image" the specimen and gain information as to its structure and composition.

A beam of electrons is generated by the thermionic electron gun. The tungsten filament (cathode) inside the gun is heated electrically until a stream of electrons is produced and a negative electrical potential (-500 V) is applied to the Whenelt Cup, which helps in directing these electrons towards the axis (horizontal center) of the microscope. This results in a collection of electrons in the space between the filament tip and Whenelt Cup and is called space charge. When a positive electrical potential (25 – 100 KV or more) is applied to the anode, these electrons get accelerated (velocity more than 200000 Km/sec at 100 KV), acquires extremely high energies and very short wavelengths (0.05 Å units) and are propelled down the column of the microscope. These electrons are further confined and focused using a system of electromagnetic lenses and metal apertures into a thin, focused, monochromatic beam, which is focused onto

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the sample using a magnetic lens. The interactions occurring inside the irradiated sample, affects the electron beam and the effects are detected and transformed into an image.

Unlike the light microscope, electron microscopes have electromagnetic lenses, each lens consisting of a hollow cylindrical shroud of soft iron containing numerous coils of copper wires to energize the magnetic gap between the pole pieces. Normally the instrument has 3 lens systems, the condenser lens system, objective lens system and the image lens system.

TEM works much like a slide projector, except that they shine a beam of electrons (like the light) through the specimen (like the slide). Whatever part is transmitted is projected onto a phosphor screen for the user to see. The "Virtual Source" at the top represents the electron gun, producing a stream of monochromatic electrons. This stream is focused to a small, thin, coherent beam by the use of condenser lenses 1 and 2. The beam is restricted by the condenser aperture (usually user selectable), knocking out high angle electrons (those far from the optic axis, the dotted line down the center). When the beam strikes the specimen, parts of it are transmitted and this transmitted portion is focused by the objective lens into an image. Optional Objective and Selected Area metal apertures are used to restrict the beam thus enhancing the contrast.

This image is passed down the column through the intermediate and projector lenses, being enlarged all the way, together achieving very high magnification and resolution to the order of about 2Å units. The image strikes the phosphor image screen and light is generated, allowing the user to see the image. The darker areas of the image represent those areas of the sample that fewer electrons were transmitted through (they are thicker or denser). The lighter areas of the image represent those areas of the sample that more electrons were transmitted through (they are thinner or less dense). The images thus formed are recorded on the photographic film.

2. Specimen processing

2.1. Sampling

Soon after the death of the organism, post mortem changes will takes place making the tissue unsuitable for ultra structure studies. Hence for electron microscopy, always live animals are preferred. The animals are sacrificed, the desired tissues/samples dissected out and immediately placed in cold fixative. The desired size of the tissue to achieve proper fixation is about 1 mm. Small animals and larvae of less than 2 mm size are fixed in ice-cold fixative as a whole in live condition. The sample vial should be labelled properly.

2.2. Fixatives

Fixatives help to preserve the structures in the living cell and prevent the changes induced by autolysis. There is no single ideal fixative and so a combination of fixatives is preferred depending on the type and nature of the tissues. In electron microscopy, 2 – 4 % Gluteraldehyde is used as the primary fixative which is excellent in fixing nucleic acids, nuclear proteins and carbohydrates but not lipids. Poor contrast and slow

penetration are the limiting factors of gluteraldehyde fixative. Osmium tetroxide is used for secondary fixation. It acts as both fixative as well as stain, fixes nucleic acids, carbohydrates and lipids and provides contrast and fast penetration. The combination of gluteraldehyde and osmium tetroxide as primary and secondary fixatives, gives the desired results in contrast and resolution.

Fixatives are prepared in a suitable buffer for two reasons, to maintain the pH (7.2 to 7.4) and to maintain the osmolality, in order to minimize the swelling or shrinkage of the tissues which may otherwise lead to artifacts. The most commonly used buffer is Sodium Cacodylate buffer.

2.3. *Primary fixation*

Tissues are fixed in 2 to 4% Gluteraldehyde in 0.1 M Cacodylate buffer (in the case of marine species, 0.5 to 3% NaCl or sucrose can be added to the fixative). For proper penetration of the fixative, the tissues should not exceed 1 mm in size. Fixation is carried out for 4-6 hrs (varies depending on the nature of the tissues), at 4°C. After fixation, the fixative is drained and tissues washed thrice (15 mts each) with buffer. In case of larger tissues, further trimming is done if required and washed with fresh buffer.

2.4. *Secondary fixation or post fixation*

For secondary or post fixation, the washed tissues are transferred to 1% Osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer, kept for 1- 2 hrs at 4°C (above 4°C, OsO_4 disintegrates). OsO_4 treatment turns the tissues black. OsO_4 is drained and tissues washed two to three times with buffer, for 15 min each, or until free of a black precipitate formed from excess OsO_4. Samples can be stored in buffer under refrigeration until further processing is desired.

2.5. *Dehydration*

Dehydration is done through graded alcohol or acetone series to remove the water from the tissues.

Dehydration can be done as follows

30% Acetone (analar)	two changes, 15 mins each at 4°C
50% Acetone (analar)	two changes, 15 mins each at 4°C
70% Acetone (analar)	two changes, 15 mins each at 4°C, (tissues can be stored in 70% acetone indefinitely, until further processing)
80% Acetone (analar)	two changes, 15 mins each at 4°C
90% Acetone (analar)	two changes, 15 mins each at 4°C
95% Acetone (analar)	two changes, 15 mins each, 4°C
100% Acetone (analar)	two changes, 15 mins each, 4°C
100% Acetone (analar)	two changes, 30 mins each at room temperature
Propylene oxide	two changes of 15 mins each at room temperature

2.6. Infiltration and embedding

Fixed and dehydrated tissues are infiltrated with liquid plastic resins and then cast into blocks. The purpose of embedding is to allow future ultra thin sectioning of the material. Commercially available Plastic resins like Epon or Spurr are used for embedding. The media is mixed as per the instructions under a fume hood. Prepare fresh media 2-3 hrs. prior to use, as it will absorb the water vapor from the surroundings and the components will begin to polymerize. Mixture of embedding medium (Spurr's medium) and acetone is prepared in various grades (mix. A – medium and acetone in the ratio 1:3, mix. B - medium and acetone in the ratio 2:2 and mix. C - medium and acetone in the ratio 3:1) and the tissue kept in each for 1-2 hrs each or as specified (period varies with the medium used) for infiltration. For embedding, medium is prepared as instructed, poured into readymade moulds made of plastic or silicon rubber and infiltrated tissues transferred to it, taking care not to trap any air bubbles. The moulds are then kept in an incubator at 70°C for 12-24 hrs.

Each tissue with reference to the experimental objective requires an evaluation of the methods, subjected to a careful examination of pertinent literature. There is no schedule that will work for all tissues and conditions.

2.7. Trimming

The resin blocks are trimmed to remove the unwanted areas using a glass knife fitted to an Ultramicrotome.

2.8. Sectioning and staining

To achieve high resolution for electron microscopy, the sections should be very thin (60 nm) and are prepared using an ultramicrotome. The resin blocks are trimmed using glass knife fitted to the Ultramicrotome. Standard procedures are followed for obtaining semi thin and ultra thin sections for light and electron microscopy respectively. Semi thin sections are first taken, stained and observed under a light microscope for determining the area for ultra thin sectioning. The blocks are again trimmed and ultra thin sections taken. These sections are floated on distilled water, stretched to remove the wrinkles and collected over the matty/dull surface of the copper or nickel grid.

2.9. Staining

Double staining with Uranyl acetate and Lead citrate is employed for routine electron microscopy studies. The sections are first stained with Uranyl acetate . A drop of Uranyl acetate (saturated solution in 50% ethanol) is taken on a clean glass slide and the grid with the section side down is kept on to the stain drop and is covered with any opaque object to ensure darkness to carry out the staining effectively. After 10-15 mts, the grid is taken out and washed 3-4 times in double distilled water (ensuring that the sections are not washed away) and dried with a filter paper. The grids are then stained with Lead citrate for 1-4 mts., washed well and dried. In the case of particulate

specimen, the specimen is taken on formvar-coated grids, subjected to negative staining using 1-3% Phosphotungstic acid and dried.

2.10. Observation and photography

The grid carrying the stained section is loaded into the electron microscope, the image observed and recorded on photographic plates/film. In order to study and interpret EM results one has to have a thorough knowledge about the ultrastructure of the normal cells and the pathogen.

2.11. Scanning electron microscopy

The scanning electron microscope, like the TEM consists of an electron optical column, a vacuum system and electronics and works under the same principle as that of the TEM. The electron gun produces an extremely fine beam of electrons, which are focused into a fine spot less than 4 nm on the specimen and scanned in a rectangular raster over the specimen. The secondary electrons produced by the interaction of the electron beam with the specimen surface as well as the backscattered primary electrons (depending upon the topography) are detected by a suitable sensor/detector. The signals from the detector are electronically amplified to modulate the brightness of a Cathode Ray Tube (CRT) so as to produce an image, which can be recorded photographically.

2.12. Specimen preparation for SEM

The specimen is first fixed with gluteraldehyde as in the case of TEM and washed well in buffer. Post fixation with OsO_4 is optional. The specimen is subjected to dehydration using ascending grades of acetone as in TEM processing. The dehydrated specimen for SEM has to be dried without causing any shrinkage. Except in the case of fine particulate specimen, critical point drying or freeze drying is usually preferred for drying SEM samples. The dried specimen is then coated with a thin conductive metal film (Gold, Palladium etc.) using an ion coater to prevent charging artifacts and to stabilize the specimen mechanically.

Presently, variable pressure SEM's are available which can operate without high vacuum thus avoiding the time consuming specimen preparation techniques as well as reduce specimen damage caused during coating.

2.13. Benefits

The most important among the benefits offered by the electron microscope is undoubtedly the very high resolution (as low as 0.1 nm) and magnification (up to 1000,000X) in TEM and 0.4 nm resolution with a magnification of up to 800,000X in SEM. Since timely and accurate diagnosis forms the first step in the health management of farmed fishes and shellfishes, the right diagnosis defines the very success of disease control. Though an array of techniques is presently available for the diagnosis of various fish and shellfish diseases, each have their own advantages and limitations. Factors like high cost of operation and infrastructure need for skilled technical personnel,

laborious and time-consuming procedures, thorough knowledge needed for interpretation etc. restricts the use of electron microscopy as a routine diagnostic tool. It can be used as a confirmatory diagnostic method for many of the existing and emerging diseases, especially of viral origin. Electron microscopy is an indispensable tool in the field of disease research and development of techniques for diagnosis.

Further reading

Electron Microscopy in Biology, Vol. 1 & 2, Edited by J.D. Griffith, 1982. John Wiley & Soas Inc., U.S.A.

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