Combined light and electron microscope in routine histopathology

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SUMMARY We report our experience with a prototype combined light and electron microscope (the LEM 2000) with particular reference to its application to routine surgical histopathology. We found its major advantages over conventional transmission electron microscopes were due to the large grid size (7 mm diameter), low magnification capacity (× 250), and the built-in microprocessor for recording areas of interest. These features combine to reduce sampling errors and greatly facilitate orientation and relocation of fields of diagnostic importance.

A new type of transmission electron microscope, the combined light and electron microscope (LEM 2000) has recently been developed by International Scientific Instruments Ltd. The Histopathology Department of this hospital has used a prototype LEM for three months, this paper being an evaluation of the instrument and associated techniques of specimen preparation. We have specifically tested possible applications of the LEM to routine surgical histopathology. Initial details of the instrument suggested that its main advantage over other transmission electron microscopes would be the opportunity to observe the same specimen in both the light and the transmission electron modes, combining selective colour staining with high resolution microscopy in the electron mode. What had not been foreseen was that the larger grid size (7 mm diameter as opposed to the conventional 3 mm diameter) combined with the low magnification capacity (× 250) and the microprocessor for recording areas of interest, would prove so useful in overcoming sampling difficulties and problems with orientation and relocation of key fields. Although other instruments have similar low magnification capacity, no other instrument, as yet, has the ability to accept a large grid.

Material and methods

Specimens were prepared using a range of tissues of varying sizes measuring from 1 mm square to 5 mm square by 1 mm thick. Tissues were processed according to the general principles involved in preparing specimens for electron microscopy. Time schedules were adjusted to allow for larger pieces of tissue. The schedule used for a typical specimen is shown in Table 1. Tissues were embedded using the Sorvall JB4 embedding system.

Table 1 Processing schedule

<table>
<thead>
<tr>
<th>Step</th>
<th>Time (h)</th>
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<tbody>
<tr>
<td>1 Fix 4% glutaraldehyde in cacodylate sucrose buffer.</td>
<td>1-2</td>
</tr>
<tr>
<td>2 Wash cacodylate sucrose.</td>
<td>1-2</td>
</tr>
<tr>
<td>3 1% osmium tetroxide in cacodylate buffer.</td>
<td>1-2</td>
</tr>
<tr>
<td>4 Wash cacodylate sucrose buffer.</td>
<td>0-3</td>
</tr>
<tr>
<td>5 Saturated uranyl acetate (aqueous).</td>
<td>1-2</td>
</tr>
<tr>
<td>6 70% alcohol.</td>
<td>0-5</td>
</tr>
<tr>
<td>7 90% alcohol.</td>
<td>0-5</td>
</tr>
<tr>
<td>8 Absolute alcohol (three changes).</td>
<td>0-5 each</td>
</tr>
<tr>
<td>9 Propylene oxide: Taab resin (1:1).</td>
<td>1</td>
</tr>
<tr>
<td>10 Taab resin (three changes).</td>
<td>24-48</td>
</tr>
<tr>
<td>11 Embed. Fresh resin and polymerise. 70°C.</td>
<td>24</td>
</tr>
</tbody>
</table>

Agitation of tissue if possible at steps 9 and 10.
*Infiltration of resin carried out at 40°C.

Sections were cut on a JB4 A microtome using 45° angled knives. Section thicknesses ranged from 1-0 µm to 0-1 µm. If possible, block faces approximately 6 mm in diameter were cut including a border of excess resin. It was found that excess resin was necessary to aid adherence of the section to the grid. Sections were cut on to distilled water and stretched by holding them a brush soaked in xylene. Sections were picked up by bringing the 7 mm diameter LEM grid underneath at an angle of 45°. Picking up sections by laying the grid on top and using surface tension was found not to work because of the increased weight of the large sections. Drying of the sections was facilitated by placing a piece of
filter paper against the side of the grid and then holding over a hot plate. Wet grids could not be laid directly on to filter paper because sections often sagged through the grid spaces, sticking to the paper. Alternatively, sections could be cut on to smaller conventional 3 mm grids as the LEM can accommodate both sizes. Sections of various thickness were stained with Reynold's lead citrate, various light microscopical stains, or with both techniques. Before some light staining techniques could be carried out the resin was first etched with sodium ethoxide for one to three seconds depending on section thickness. With basic dyes such as toluidine blue, methylene blue and basic fuchsin, this was not necessary. If the sodium ethoxide treatment lasted too long sections fell apart in the electron beam. Sections from tissue fixed in osmium tetroxide were also treated with 1% periodic acid for 10 min to improve light microscopic staining. Staining techniques attempted were toluidine blue, methyl violet, haematoxylin and phloxine, periodic acid Schiff, alcian blue, Giemsa, Jones' hexamine silver.

Sections stained for light microscopy and viewed in an electron microscope present new problems. Ultramicroscopic stain deposits, undetectable by light microscopy, can appear as large obstructions at electron microscopic level, impeding viewing of the specimen. To avoid this, all stains were carefully filtered.

Staining techniques were carried out in micron embedding capsules or alternatively on a drop of stain on a glass slide. Using the latter method, heating of the stain was easily facilitated by placing the slide on a hot plate. Drying of sections after staining was carried out as before. Human material we examined in the LEM included renal biopsies, liver biopsies, lymph nodes, and lymphomas and various tumours—particularly some thought likely to contain neurosecretory granules or contractile filaments.

**Results**

Post-fixation and staining with osmium tetroxide and uranyl acetate was found to give the best detail of fine structure and contrast for visualisation. Specimens fixed only in glutaraldehyde were more difficult to examine even when stained with uranyl acetate. The block was stained with uranyl acetate rather than the cut section because of ease, and also because we found that it was virtually impossible to stain with toluidine blue a section already stained with uranyl acetate.

Sections more than 0.5 µm thick gave a very clear light microscopical picture although electron microscopy was difficult to interpret. Reducing section thickness to 0.5 µm-0.25 µm made light microscopic staining more difficult: some techniques gave insufficient density of staining for interpretation. Toluidine blue was the most informative stain at this thickness. Electron microscopic examination of sections in this thickness range produced a clear enough image for all cytoplasmic organelles to be recognised.

With the reduction in section thickness to 0.25 µm-0.1 µm (Figs. 1 to 4) a considerable increase in electron optical resolution was achieved, sacrificing some light optical contrast. Limited light microscopy could be carried out at the thicker end of this range with the toluidine blue technique (Figs. 1 and 3). One way of obtaining acceptable light microscopy and good electron microscopy was found to be to mount 0.25 µm and 0.1 µm thick sections side by side on the grid and then stain by the combined toluidine blue/lead citrate method (Fig. 3). We could achieve good light microscopy on the thicker section and good electron microscopy on the thinner section. The limit of electron optical magnification with this range of thickness was × 20,000. One other finding of some interest at this section thickness was that pretreatment with 0.5% potassium permanganate (acid) followed by 1% oxalic acid enhanced the toluidine blue staining. However, on subsequent examination in the electron microscope, there seemed to be some loss of clarity. Possibly the lead had been washed out.

![Fig. 1 Light microscopy of 0.2 µm thick section processed as in Table 1 and stained by toluidine blue/lead citrate. Renal biopsy, membranous glomerulonephritis. Photo of section on the grid × 70.](http://jcp.bmj.com/)

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Fig. 2 Transmission electron micrograph of part of the same glomerulus in the same section illustrated in Fig. 1. Despite the unconventional thickness of the section the characteristic diagnostic epimembranous deposits of membranous glomerulonephritis are obvious × 1720.

Fig. 3 Light micrograph of thick (0.25 μm) and thinner (0.1 μm) serial sections of a renal biopsy mounted side by side in the 7 mm diameter grid. Sections stained by combined toluidine blue/lead citrate method × 3.

Some conventional electron microscopy was carried out with sections thinner than 0.1 μm (Fig. 5). The instrument was capable of studies to levels of resolution equal to that considered to be conventional for thin sections of tissue—that is, 1/10 of the section thickness (Cosslett's law). Suggested uses of sections of different thicknesses are summarised in Table 2.

Fig. 4 Low power transmission electron micrograph (0.2 μm thick, stained by combined toluidine blue/lead citrate method) of follicular lymphoma. The follicle occupies the upper right half of the field. Such areas were easily found in the 5 mm square sections, but very difficult to find in 1 mm square sections of the same specimen × 600.

Fig. 5 Conventional thickness (0.05 μm) and staining (uranyl acetate/lead citrate) of section of spleen in Hairy Cell Leukaemia showing typical Hairy Cell. The LEM functioning as a conventional transmission electron microscope × 6980.
Table 2  Specimen thickness evaluation

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>Section thickness</th>
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<tbody>
<tr>
<td></td>
<td>0.7-0.5 μm</td>
</tr>
<tr>
<td>LM</td>
<td>Suitable for</td>
</tr>
<tr>
<td></td>
<td>a wide range</td>
</tr>
<tr>
<td>EM</td>
<td>up to 3000</td>
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</table>

It was noticed that under the electron beam the light microscopy stain was "burned off" leaving areas of unstained resin when re-examined in the light microscope. The section could not be re-stained due to further polymerisation of the resin in the heat of the beam. Silver staining techniques could not be carried out with the section on the grid as the solution attacked and dissolved the copper grid. The section had to be first stained and then mounted.

The main advantage of the LEM in diagnostic histopathology only became apparent when we began to concentrate on using the instrument as a transmission electron microscope, exploiting its large grid size (7 mm as opposed to the conventional 3 mm), low magnification capacity (×250) and microprocessor for recording areas of interest. We found, for example in focal glomerulonephritis, that the larger number of glomeruli available for examination and easily reidentifiable made electron microscopic diagnosis of this condition much easier and more reliable. In the electron microscopic examination of lymphomas we found orientation very much less of a problem than with conventional grids and in follicular lymphomas we could easily find and examine neoplastic follicles (Fig. 4).

In the investigation of the histogenesis of tumours thought likely to be rhabdomyosarcomas or likely to contain neurosecretory granules—a larger section, with low power scanning capacity and the ability to record and relocate areas of interest, obviously improved the chances of making a positive diagnosis. The examination of pieces of intestinal mucosa and pancreas was also greatly facilitated on larger pieces of tissue—orientation was much easier and islets simple to find. Pieces of liver could be examined faster and low power scans for HBsAg and HBcAg in hepatocyte cytoplasm and nucleus respectively were much more readily performed.

In other words, sampling and orientation problems were greatly reduced in the LEM. When we wished to have a second look at a section at a later date, because the 7 mm diameter grid would only fit into the grid holder in one position the section was identically orientated every time it was placed in the instrument. With the co-ordinates automatically recorded on every photograph, areas of interest were readily relocated.

Discussion

In the short time we had the use of the prototype LEM we certainly did not study its potential exhaustively. We probably underestimated the advantages of its capacity to view the same section by light and electron microscopy. For example, we did no histochemical nor immunoperoxidase work and as the reaction products of these techniques can be visible by both light and electron microscopy, this would seem an ideal area in which to use the instrument. Also, the examination of brain tissue with the use of silver staining techniques would seem another area in which much might be achieved.

However, our aim was to develop specimen preparation techniques and to evaluate the instrument as a tool in diagnostic histopathology. The various technical problems encountered and our solutions to these are reported. Many of the techniques we tried had to be modified as the requirements and the potential of the instrument were new to us, but none of the problems seemed insurmountable and most were easily solved. We personally think that the main advantages of the LEM over conventional transmission electron microscopes in the field of diagnostic histopathology stem from the large grid size. Furthermore, the capacity of the instrument to view larger sections could be useful if only a very limited amount of tissue were available for microscopic examination. In such circumstances it is obviously an advantage not to have to divide a specimen into parts for electron microscopy and light microscopy. The whole specimen can be embedded and a section from the whole face of the specimen can be examined in the LEM.

The microprocessor system for relocating areas of interest is certainly a convenience although 3 mm diameter “finder” grids perform the same function in conventional electron microscopes. The large LEM grid is a finder grid, but we found the relocating system invaluable in dealing with this larger area. We know there are those who would prefer to retain separate dedicated instruments for light and electron microscopy. One is compromising to some extent with a combined instrument such as the LEM, but the only compromise as far as the electron microscopist is concerned is the upper limitation in magnification (×45,000). This, however, has to be balanced against advantages such as the potential of large sections and the ability to look at exactly the same section by light and electron microscopy.
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With the current difficulties of both justifying and financing electron microscopes within the health service, the introduction of the LEM offers a new view. Semithin microtomes can be used for section cutting; ultramicrotomes are not essential—so making specimen preparation cheaper and simpler. The operation of the LEM does not require a high degree of manipulative skill and it does not require a darkened environment.

In summary, we find the LEM 2000 to function as a good transmission electron microscope. Its capacity to take large grids and sections reduces sampling error and helps with orientation. The built-in microprocessor records and relocates areas of interest and photographs are all automatically numbered and have the co-ordinates of the field marked on them. One has the opportunity to observe the same section by light and electron microscopy, or to examine parallel mounted serial thick and thin sections on the same grid to optimise light and electron definition.

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References

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