

Correlative Light Electron Microscopy Using High Pressure Freezing

CLEM: Combining the Strengths of Light and Electron Microscopy

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Fig. 1: Schematic model of a CLEM experiment. In a modern CLEM experiment a sample is first studied live under the light microscope (Green cells). A structure inside the cells can be followed (arrows). When an interesting event occurs the sample is fixed and processed for electron microscopy. The same cell is traced back in the EM (grey image) and the structure and event of interest can be studied at high resolution.

In recent years light microscopy studies have been dominated by live cell imaging while electron microscopy has been used for high-resolution studies. Latterly, there has been increasing interest in combining these techniques. This combination is called Correlative Light Electron Microscopy (CLEM). Due to the high resolution made possible by electron microscopy, artefacts induced during preparation of a sample can, however, also be clearly seen. The Leica EM PACT2 with RTS is a high pressure freezer designed for CLEM experiments to allow excellent preservation of ultrastructure, thus avoiding such artefacts.

With the emergence of Green Fluorescent Protein (GFP) in the 1990s the interest of live cell imaging studies has been tremendous. This, coupled with faster and more sensitive detection systems on microscopes provides even greater opportunities. Life science research has learned such an incredible amount from these live cell imaging studies that it is hard to imagine what research would be like without these tools.

The field of electron microscopy has not fully locked onto the momentum of the light microscopy wave, but in recent years it has become evident that the resolution of the light microscope is in some cases limiting in resolving the scientific question. Therefore, there has been increasing interest in developing techniques that combine the live cell imaging aspect of GFP with the high resolution of EM (Fig. 1). GFP is not directly visible in the EM (electron microscopy) but can be visualised using antibodies or by photo conversion. However, such methods are based on samples that are chemically fixed at room temperature. It is well documented that this can introduce artefacts. Such artefacts are not visualised at the LM (light microscopy) level since there is either no fixative present yet (live) or when fixed samples are studied the artefacts are below the resolution of the LM.

However, at the EM level they become apparent and that is exactly where we want to study structures at

high resolution and in precise detail. There is an alternative fixation method that is based on physical fixation, namely, cryofixation. It fixes cells much faster and unlike chemical fixation is non-selective. High Pressure Freezing (HPF) is nowadays the most reliable method for cryofixation of cells and tissues. A sample is put under high pressure (2000 bar) and milliseconds afterwards the sample is sprayed with liquid nitrogen. This prevents the formation and growth of ice crystals and fixes samples instantly with up to about 200–300 μm depth of well-preserved, vitreous sample.

High Pressure Freezing with new time resolution

HPF machines were not originally designed for these types of experiments, the major constraint being the time it takes to study a sample under the LM, spot an interesting event and then transfer that sample into an HPF machine to fix it. This would take at least 30 seconds for a very experienced user. By that time the structure of interest would have already disappeared (Fig. 1).

So, Leica Microsystems, together with Dr. Paul Verkade (School of Medical Sciences, University Walk, Bristol, UK), set out to develop a tool that would

be able to carry out CLEM experiments with HPF but with a time resolution of less than 5 seconds. The end result is the Leica EM PACT2 with EM RTS (Fig. 2). It is mobile, so it can be moved to any light microscope. The RTS stands for Rapid Transfer System. This system consists of a rapid loader that contains the sample (Fig. 3) and the actual transfer system as an attachment to the HPF machine. After insertion of the rapid loader into the RTS, the sample is automatically enclosed and shoots along a rail into the Leica EM PACT2 to be frozen. This sequence only takes 2.4 seconds. This leaves enough time for the scientist to take the rapid loader from under a light microscope and put it into the RTS. Such a movement can easily be done within 1–1.5 seconds and thus results in an overall time resolution of about 4 seconds. The frozen sample can now be processed for electron microscopy.

New standard of freeze substitution

In most instances this will involve freeze substitution, where the frozen water is removed from the sample

with solvents such as acetone prior to resin infiltration and polymerisation at low temperature. Here too, Leica Microsystems has recently made it much easier for the scientist with the introduction of the Leica EM AFS2 with EM FSP that allows for automatic exchange of all the freeze substitution chemicals. Figure 4 shows an example of such an experiment. Epidermal Growth Factor (EGF) was coupled to quantum dots. Quantum dots are both fluorescent and electron dense and therefore excellent markers for CLEM studies. The EGF has been internalised for 30 minutes and is present in multi-vesicular bodies (MVB). The MVB appear as a dynamic structure with lots of extensions appearing and disappearing over time (Fig. 4c).

When another extension was seen appearing the sample was taken and immediately frozen. The same cell is retraced and the structure of interest can now be visualised at high resolution, showing the connections. In conclusion, the Leica EM PACT2 plus EM RTS coupled with Leica Microsystems' confocal microscopes offers life scientists an excellent solution that serves both to extend their toolbox and knowledge.



Fig. 2 and 3: The Leica EM PACT2 + RTS high-pressure freezer, suitable for standard high pressure freezing and CLEM experiments. Its operation is very easy: All you need to do is insert the rapid loader, the machine will do the rest.

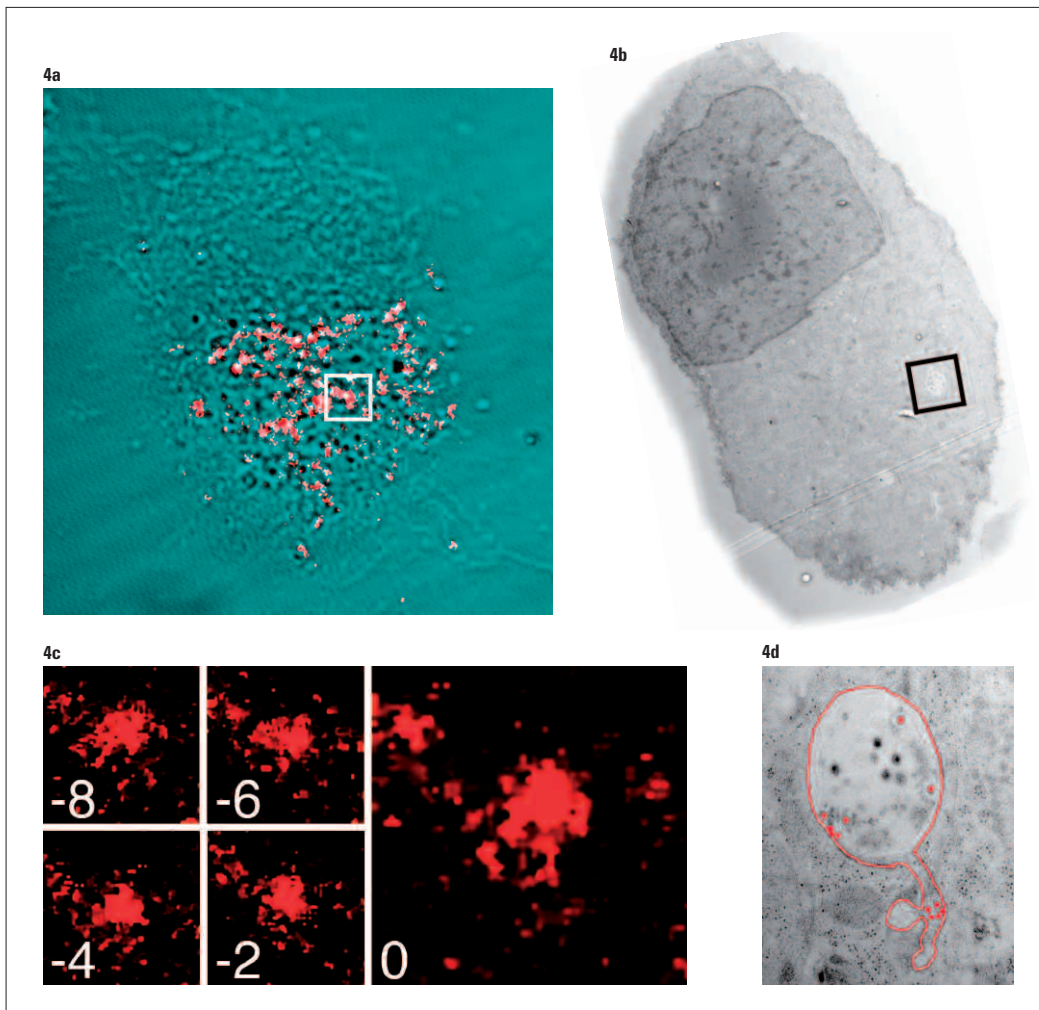


Fig. 4: Example of a CLEM experiment using the Leica EM PACT2 + RTS. A cell of interest is first studied at the light microscopy level. The DIC image of a cell of interest is overlaid with the red fluorescence of the quantum dots (Fig. 4a). The structure of interest is boxed and followed live. Fluorescence images are taken and the sequence of the last 10 seconds is shown (Fig. 4c). At time 0 seconds the rapid loader is taken and placed in the RTS. After it has been frozen and processed, the same cell is traced back (compare Figs. 4a and 4b). Note: The area of the nucleus as can be seen in the EM image is devoid of fluorescent label as would be expected. Now the structure of interest can be studied at high resolution and we see (Fig. 4d) a connection (membrane is false-coloured red) between the main MVB and the tubular extension. Both the main MVB and the extension contain Quantum dots (red dots false coloured).