Microscopy Focus

THE USE OF HIGH-PRESSURE FREEZING IN CORRELATIVE LIGHT ELECTRON MICROSCOPY

Paul Verkade

With the emergence of Green Fluorescent Protein (GFP) light microscopy has switched from mainly static immuno fluorescence images into imaging very dynamic processes. Dynamic imaging is impossible in the electron microscope but it is excellent for high-resolution studies. Combining both advantages in techniques collectively called Correlative Light Electron Microscopy (CLEM) has gained increasing interest in recent years.

New developments within this field include probe development, probe detection, and fixation. Some of these new developments will be discussed and a particular focus will be placed on the fixation process for electron microscopy.

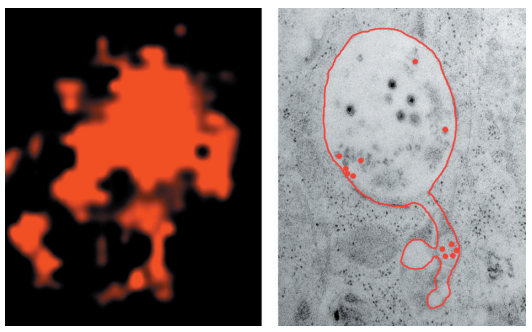
"COMBINING LIVE LIGHT MICROSCOPY WITH HIGH RESOLUTION ELECTRON MICROSCOPY IS THE REAL CHALLENGE"

Author Details:

Paul Verkade, Senior Lecturer/Electron Microscopist, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD

Email: p.verkade@bristol.ac.uk

The interest in doing live cell imaging studies has increased tremendously with the emergence of Green Fluorescent Protein (GFP) at the end of the last century [1]. Almost any self-respecting cell biology lab is now using GFP or one of its derivatives in some sort of form. It has caused a quantum leap in knowledge within the life science research and it is very hard to imagine what research would be like without these tools. Electron microscopy did not fully hook into that momentum of increased interest in microscopy techniques. But in recent years it has become evident that when light microscopy is used as the read-out assay, the outcome of such experiments is sometimes not unequivocal. The limitation in light microscopy resolution power plays a major role in these 'failures'. The emission wavelengths of the fluorophores as well as the resolution of the light microscope (LM) are within the order of 100 nm, whereas sub-cellular structures are mostly in the range of 10s of nm.



Combining live light microscopy with high resolution electron microscopy is the real challenge.

Therefore the resolution of live LM experiments is in most cases not sufficient to assign the label to specific structures or, even more important nowadays, its localisation within domains of that structure. For such questions electron microscopy (EM) is the technique of choice. There of course, the major limitation has always been that living samples cannot be observed so that it is impossible to deduct the sequence of events (the history of a structure) from EM alone. Ideally one would like to study a sample live with the resolution and additional spatial information of the electron microscope (nanometers). Such a microscope is at the moment technically impossible (may be even never). Therefore, parallel with the development of all the new LM techniques and tools there has also been an increasing interest in so-called Correlative Light Electron Microscopy (CLEM) techniques. In such imaging techniques a sample is first analysed (live) at the LM level, and then subsequently further studied at higher resolution at the EM level. Ideally CLEM techniques would combine the advantages of high time resolution live imaging and multi-colour probes at the light microscopy level, with the analysis at the high resolution of the electron microscope in 3D (electron tomography).

Most CLEM techniques have focused on the detection of the probe for both LM and EM, with GFP being the most obvious choice. There is however at least one major drawback to the ways the GFP is being used for the electron microscopy part of the technique: GFP is not directly visible in the electron microscope. Hence, it needs additional processing to visualise the GFP. Antibody labelling [2] or photo conversion [3] and the subsequent formation of an electron-dense (DAB) precipitate can achieve this. Other CLEM techniques have tried to overcome that problem by developing a probe that is both 'visible' in the light microscope and the electron microscope. The FlAsh / ReAsh [4], (currently called Lumio™) uses a very short cysteine-rich expression tag that is recognised by and binds to the fluorescent probe. The red variant is again able to form a DAB precipitate and can thus be used for CLEM experiments. The toxic nature (arsenic) and the background staining of the probes have so far prevented the technique to fulfil all the promises at its emergence. In addition, a major drawback to the techniques mentioned above (GFP and FlAsh/ReAsh) is the use of DAB precipitation. It can diffuse and obscure the precise size and / or location of the actual probe [5]. A new emerging tool within life science research is the quantum dot.

These metal alloys combine properties for fluorescent imaging and electron microscopy since they are brightly fluorescent and due to the presence of the metals also electron dense. These probes are being used more and more for CLEM experiments [6]. One drawback of the quantum dots is that, being alloys of metals, they are in principle toxic [7]. In order to prevent those toxic effects an insulating protein coating is used to cover the quantum dots which depending on the coating reduces or prevents the toxic effects [8]. This protein coating however also offers the possibility to overcome a different challenge.

Cells themselves can't make quantum dots like they can with GFP. In order to use them in such a setup one would need to tag the quantum dots with a specific recognition signal. This can be attached to / incorporated in the protein coating (quantum dots are commercially available bound to antibodies or streptavidin). The functionalised quantum dot should then cross the plasma membrane [9], give no background or toxic effects, and then recognize a short peptide expressed on the protein of interest (like the Lumio™ tag)[10]. This is a very interesting strategy that is pursued by different labs but its technical success is very unpredictable. Moreover from personal experience it was found that the imaging properties of the quantum dots for the electron microscope, and hence CLEM, are not optimal. Since they mainly consist of Cadmium and Selenium, the atom numbers are not high enough to deviate electrons very much from their path and hence do not appear as black spots but more greyish. The staining agents used (Osmium, Uranium, Lead) are of higher atom number than those in quantum dots. One would need to incorporate heavier atoms in the quantum dots (core or shell) to allow for easier visualisation. At present one needs to compromise between visualisation of the quantum dots and optimal staining of the material. Omitting certain counter stains or extraction of material from the cytosol can achieve this. This need for this compromise is a current drawback in the use of quantum dots for (live) CLEM experiments. The major reason for doing electron microscopy in CLEM experiments is the possibility to acquire high-resolution data. The compromise will usually be at the expense of retention of ultrastructure. Samples are chemically fixed and dehydrated at room temperature. It is well documented that both those steps can introduce artifacts [11, 12]. It would make the results of CLEM studies on membrane fusion / fission etc. questionable because one could well be studying artifacts in the electron microscope. Such artifacts are not visualised at the LM level since they are below the resolution of the LM. But at the EM level they become apparent and that is exactly where we want to study structures at high resolution and in precise detail. High-pressure freezing [HPF, 13, 14] offers an alternative fixation method that is based on physical cryo fixation. HPF fixes material much faster and non-selective than chemical fixation and is currently the most reliable method for fixation of cells and tissues. In HPF a sample is sprayed with liquid nitrogen under high pressure, preventing the formation and growth of ice crystals.

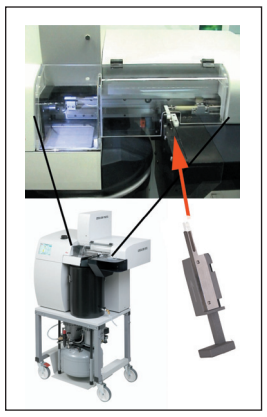


Figure 1. The EMPACT2 + RTS (top + bottom left) is a new mobile high-pressure freezer with an automated transfer attachment (Rapid Transfer System, RTS, top image) suitable for CLEM experiments. After insertion of the sample located at the tip (red circle) of the rapid loader (bottom right) into the RTS (red arrow), the RTS will automatically enclose the sample and transfer it into the high-pressure freezer where it will be cryo-fixed.

So far HPF machines were not suited for CLEM experiments. The transfer of a sample from the light microscope to the high pressure just takes too long (30 – 60 seconds, see also [15]). Together with Leica Microsystems we have recently developed an attachment to a high-pressure freezer that allows for fast transfer of a sample from the light microscope until the moment it is frozen. The high-pressure freezer (EMPACT2) is placed on a trolley so it can be placed next to any microscope. The attachment is a Rapid Transfer System (RTS, *Figure 1*) that automatically encloses a sample and transfers it along a rail into the HPF machine to be frozen. This automated sequence takes about 2.5 seconds. This leaves the time for the scientist to move the sample from under a light microscope and put it into the RTS. Such a movement can easily be done within 1 – 1.5 seconds *(Figure 2)* and thus results in an overall time resolution of about 4 seconds for a HPF-CLEM experiment. What would be the result of a HPF-CLEM experiment using Quantum dots? Quantum dots were coupled to Epidermal Growth Factor (EGF) and internalised for 30 minutes into A431 cells. The EGF-quantum dots are present in multi-vesicular bodies (MVB) that appear as dynamic structures with lots of extensions appearing and disappearing over time *(Figure 3)*.

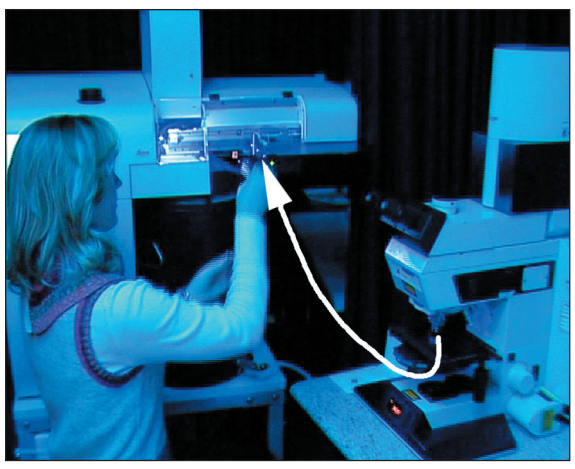
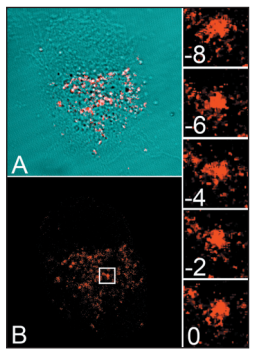


Figure 2. When the EMPACT2 + RTS is placed next to a light microscope the transfer from the sample into the high-pressure freezer (arrow) will only take about a second.

When such an 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 *(Figure 4)*.

Life science research has made some giant steps forward being able to capture the dynamic nature of cells with live cell imaging but it is realized that for some applications it has limitations. With regard to resolution the solution at the moment is CLEM, combine live LM data with high resolution EM data. With the development of new probes and better fixation techniques that are specifically designed for CLEM studies it opens up a whole new avenue of possibilities to use the CLEM technique in a variety of studies. The development of new probes that are both fluorescent and electron dense (like QD) will require further study. The focus of this research will be on probes that are able to penetrate the plasma membrane and bind to specific recognition signals that would allow live cell imaging or the development of biosynthetic molecules for LM and EM without the need of additional processing.

Figure 3. Example of the LM part of a CLEM experiment using the EMPACT2 + RTS. The DIC image of a cell of interest is overlaid with the red fluorescence of the quantum dots to be able to relocate the ROI (A). The structure of interest is boxed (B) and followed live. Fluorescence images are taken and part of the sequence of the last 10 seconds is shown. At time 0 seconds there is an extension emerging at the bottom of the big fluorescent structure, the rapid loader was taken and placed in the RTS and the sample was frozen.



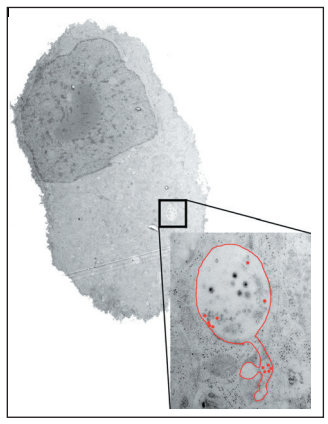


Figure 4. After processing for electron microscopy, the same cell is traced back (compare with Figure 3A).

Note: The area of the nucleus as can be seen in the EM image is devoid from fluorescent label as would be expected.

When studied at higher magnification the structure of interest can be traced back and the same extension as was observed in the light microscope can be observed (see also opening image). As discussed the quantum dots are not easily visualised and are false-coloured as red dots. It can be observed that the main MVB and the extension contain quantum dots and are still connected (membrane is false-coloured red).

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