Correlative Light and Electron Microscopy

Leica

MICROSYSTEMS

CONTENTS

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INTRODUCTION

Understanding in detail the incredibly dynamic behaviour of cells and tissues has preoccupied scientists since the dawn of microscopy in the 17th century. A structural and functional characterisation of cellular components and their elaborate interactions is one of the ultimate challenges in biology. Such a thorough understanding of these functions, however, requires both a macroscopic view of the whole cell and the ability to focus on single molecules, ideally under their natural environment. It's every scientists' dream to be able to 'zoom in' seamlessly from the whole cell to individual cellular structures or proteins, all using the same instrument.

Although this seamless approach is still in the realm of fantasy, the urge to go deeper into the microscopic world has led researchers to combine the versatility of the light microscope with the resolution power of the electron microscope to produce correlative light and electron microscopy (CLEM). This technique has opened the door to studying the interior of a single cell at a range of different scales, from the micrometre to the nanometre.

CLEM's most significant and powerful characteristic for cell biology research is an ability to study the *same cell* using two different microscopy platforms. Each cell may have to endure labelling with different probes to allow imaging with both light microscopy and electron microscopy, but this dual examination provides valuable complementary and often unique information.

This *Essential Knowledge Briefing* aims to describe the basics of CLEM, including a brief overview of the most commonly used procedures and vital information for the successful application of this technique. It also reviews potential pitfalls and problems

encountered when developing the procedures, as well as useful tricks for solving them. The briefing ends with a peak into the future, looking at what major breakthroughs could be expected over the next decade.

HISTORY AND BACKGROUND

CLEM combines the capabilities of two typically separate microscopy platforms: light (or fluorescence) microscopy (LM) and electron microscopy (EM). The advantage of LM is that it can provide wide field images of whole, often live, cells, but its resolution is limited. The advantage of EM is that it can provide much higher resolution images, up to molecular dimensions, but only over specific regions of a cell at a time and not in live cells. CLEM combines the advantages of both techniques, allowing scientists to spot cellular structures and processes of interest in whole cell images with LM and then zoom in for a closer look with EM. It's like studying a specific building from an aircraft: it's easier to locate the building first and then look at it through binoculars than try to find the building through binoculars. It's important to emphasize, however, that a correlative approach should only be used where there is a significant benefit over using each technique in isolation.

The concept of combining multiple microscopy systems is not a new one in biology. After the advent of EM in the early 20th century, researchers quickly realised the benefits of using several different types of microscope, each possessing different characteristics, in the same study. The oldest reference to 'correlative microscopy' dates back to the 1960s, and although these procedures cannot be classed as 'correlative microscopy' by today's standards, they provided the inspiration and drive to develop a more elegant system.

The ability to use LM and EM to image the same cell evolved over the following years and, by 1987, the first compilation of CLEM techniques (*Correlative microscopy in biology. Instrumentation and methods* edited by MA Hayat) was published. In 20 chapters, the book provided detailed examples of correlative microscopy

possible at the time, marked by time-consuming chemical fixation of cells and limited choice of probes for immunostaining. For example, Swedish researchers Erik Wilander and Monalill Lundqvist described a sequential technique using immunostaining followed by treatment with silver nitrate after cell fixation in a formaldehyde solution. Another example by Canadians Dieter Geissinger and Peter and Lorraine Rhodes involved using LM to identify regions of interest in microtome sections of striated muscle, followed by examinations with scanning and transmission electron microscopes after treatment with osmium tetroxide and epoxy resin embedding.

The panoply of available LM and EM probes increased over the years – for example, fluoronanogold was introduced in 1998. It wasn't until 2000, however, that Alexander Mironov's team from the Institute of Pharmacological Research in Italy revolutionised CLEM when they used green fluorescent protein (GFP) for the first time.

Mironov and his team were looking to assess the morphology of protein carriers shuttling between the Golgi apparatus and the plasma membrane. So, rather ingeniously, they tagged a carrier protein called vesicular stomatitis virus G protein (VSVG) with GFP and then chemically fixed transfected cells at time-specific points. The fluorescent tag was further labelled with immunoperoxidase for EM analysis, allowing the researchers to assess in detail the structural and dynamic characteristics of the organelles involved in protein trafficking.

Although few research groups embraced CLEM in the early years, it has now built a considerable following, with the numbers of studies increasing exponentially. CLEM is maturing into a

sophisticated and attractive method for answering fundamental research questions about cellular processes like membrane trafficking, signalling and cell division. Scientists are also discovering how it can be used for more applied research, such as diagnosing disease or developing new drugs for cancer treatments. It has now left the comfort of research labs, as industrial applications become popular; these include assessing the structure of various foods or examining fatigue striations in metallic alloys.

In biology, CLEM has made it possible to zoom in on dynamic cellular events as they occur and even to visualize single organelles and how they interact in individual cells. However, demand from scientists to study a wide range of different mechanisms in a variety of different cells has inevitably led to the development of many CLEM procedures. In fact, CLEM should not be seen as a single technique, but more realistically as a collection of techniques with a common background.

Overall, there are two main CLEM approaches, differing significantly on the emphasis they place on the use of LM or EM. The first approach uses LM and immunolabelling mainly to locate a region of interest in fixed cells before analysing them in fine detail by EM. In contrast, the alternative approach highlights the possibility of dynamic live cell imaging with LM, supplemented by observations with EM.

Ultimately, the choice of CLEM method depends on the research question. For example, for studies of membrane transport, the initial phase with light microscopy must include a live-cell step to reconstruct the dynamic process. In contrast, for detailed analysis of individual organelles, the emphasis is more likely to be on the high resolution possible with electron microscopy.
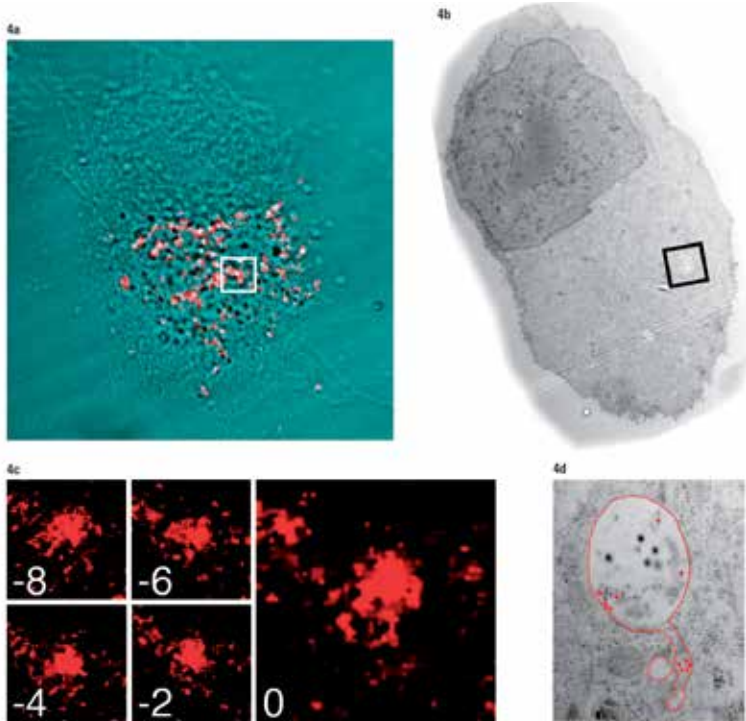
Example of a CLEM experiment using the Leica EM PACT2 + RTS. A cell of interest is first studied at the light microscopy level. The DIC image of a cell of interest is overlaid with the red fluorescence of the quantum dots (Fig. 4a). The structure of interest is boxed and followed live. Fluorescence images 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 from 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).

Courtesy: Dr. Paul Verkade (School of Medical Sciences, University Walk, Bristol, UK)

In a conventional CLEM study, the sample is first labelled with fluorescent probes and then imaged with LM, allowing interesting structures and processes to be identified. These fluorescent probes can include the fluorescent dyes linked to antibodies used in traditional immunostaining, but can also include more modern examples such as fluorescent proteins, quantum dots (Qdots) or enzyme-based probes such as horseradish peroxidase (HRP). Next, the sample is immobilized by chemical fixation or freezing, labelled with immunogold or another EM probe, and then embedded prior to analysis of the specific structures and processes of interest by EM.

Not all available markers appropriate for LM can survive the harsh processes involved in preparing a sample for EM. Many organic dyes and fluorescent proteins quench when moved from the warmth of the aqueous solutions for live-cell imaging into solvents and fixative solutions. Despite this limitation, the use of two or more fluorescent markers in different colours is now commonplace, which is ideal for the analysis of interactions between different cellular structures.

While in some cases chemical fixation may be the best approach, cryogenic methods are currently the gold-standard in sample preparation for CLEM and believed to cause less damage than the traditional chemical approach. Most researchers agree that, although not entirely artefact-free, freezing methods are faster and less distorting than conventional fixation with glutaraldehyde and osmium tetroxide. Cellular activity is frozen within milliseconds, compared with minutes for chemical fixation. The goal of cryo-fixation is to convert all the water to vitreous ice, which occupies the same volume as water, unlike crystalline ice. In reality,

the formation of small ice crystals is inevitable, but doesn't cause a major problem.

Traditionally, the material used for embedding samples for CLEM has been paraffin or epoxy resin, but less hydrophobic materials, such as LR White or Lowicryl, are becoming increasingly popular. This approach has been named plastic embedding and its main advantage is the ability to produce ultra-thin or semi-thin sections. This cannot be achieved with other materials and allows for an increased level of detail over thick sections.

Despite the incredible results obtained with this technique, scientist ideally want to observe the dynamics of live cells. For a long time the standard CLEM technique precluded this, but in 2008 Judith Klumperman's team from the University Medical Center Utrecht in The Netherlands was able to combine live-cell data with EM. In the first demonstration of this method, they monitored the dynamics of specific endosomes by tracking the lysosomal protein LAMP1 tagged with GFP. They then used anti-GFP antibody EM-labelling to confirm the LAMP1 content of tracked endosomes. A more recent example comes from the University of Pittsburgh School of Medicine in the USA, where P. Zhang's team used CLEM to study HeLa cells infected with HIV-1. Live cells were characterized first by confocal microscopy, and then analysed by cryo-electron tomography for structural details.

IN PRACTICE

Correlative microscopy can provide extraordinary results, but it's a demanding and time-consuming technique, and mastering its several steps can prove difficult. However, recent developments in both the field of live-cell imaging and EM, as well as the emergence of powerful new staining techniques, are transforming CLEM from a 'heavy' technique only available to a handful to research groups into a more user-friendly procedure that should appeal to an increasing number of scientists.

The main trick with CLEM is to stain the structure under study in a manner suitable for detection by both fluorescence and electron microscopy. Making this more difficult is the fact that no single probe can currently be used for both LM and EM, requiring the sample to be labelled first with LM probes and then with EM probes. However, researchers are now developing ways to use LM probes to produce EM probes.

Genetically-encoded fluorescent proteins such as GFP are popular fluorescent probes for CLEM, especially when imaging live cells. The only reason why GFP is not a perfect CLEM probe is because it's not electron dense and its fluorescence is usually quenched by fixation and embedding methods. To overcome this issue, scientists have developed a technique known as GFP-recognition after photo-bleaching (GRAB). This method involves the photo-conversion of a molecule called diaminobenzidine (DAB) by the fluorescence emitted by GFP, resulting in the formation of a highly-localised electron dense precipitate that can act as an EM probe.

A similar approach utilizes the molecules fluorescein bi-arsenical hairpin-binding (FlAsH) and the resorufin red variant (ReAsH).

FlAsH and ReAsH are small, membrane-permeable fluorescent molecules that recognize a tetracysteine motif (CCxxCC) linked to the protein of interest. Although suitable for LM, neither of these two probes are visible with EM. However, ReAsH can photo-convert DAB in the same manner as GFP to form an osmiophilic precipitate distinguishable by EM.

There are some drawbacks to using DAB as an EM probe, including diffuse staining and the possibility that it will drift from its original location and spread to the surrounding region. Indeed, DAB works best when the protein of interest is encapsulated within an organelle. In this case, the dissemination may turn out to be useful, as it provides an accurate delineation of the labelled organelle, facilitating three-dimensional reconstruction.

Recently, a team of researchers from California, led by Roger Tsien, developed the first genetically encoded fluorescent protein available for EM examinations, which they called miniSOG. This is a small fluorescent protein that generates singlet oxygen when illuminated with blue light, which in turn polymerises DAB into a precipitate that is stainable with osmium and therefore can be imaged by EM. Using this approach, the authors demonstrated the localization of synaptic cell adhesion proteins both in neuronal cell cultures and intact mouse brains.

After the LM step, the specimen needs to be fixed, usually by freezing. The latest development in cryo-fixation is High Pressure Freezing (HPF), which has been used to follow highly dynamic events such as vesicular trafficking or nuclear division in the nematode *Caenorhabditis elegans* by Thomas Müller-Reichert's team at the Technical University of Dresden in Germany (see *Case study 3*). It

is now possible to capture specific events occurring in living cells as observed by light microscopy and, at the most appropriate point, cryofix the sample within seconds and analyse that particular event at high resolution with EM.

Samples can either be imaged in the vitrified state, or be freeze-substituted and embedded in resin before imaging. This procedure involves replacing water with chemical fixatives, but it's done at low temperature and is not associated with the same distortions as standard chemical fixation, which is done at room temperature.

The most popular method currently available for HPF is the Rapid Transfer System (RTS), developed by Paul Verkade at the University of Bristol, UK, for the Leica EMPACT2. Jocelyn Laporte's team from the Institut de Génétique et de Biologie Moléculaire et Cellulaire in France used this elegant approach to follow the effects of hyper-osmotic treatment. Cells were transfected with several fluorescent protein fusions and observed with a confocal microscope. They were then high pressure frozen within five seconds, and embedded and immunogold-labelled prior to study by a version of EM known as electron tomography.

Another major challenge with CLEM is accurate image alignment to ensure that the exact same cell is observed under LM and EM. This is not a straightforward process, as distortions can occur due to the inherent differences in scale between the two microscopy techniques, as well as the numerous processes that may cause physical distortions, such as shrinkage.

The most common way to keep track of the desired region of interest when changing between LM and EM is by using coverslips marked with a small grid. Crucially, these grid markings will
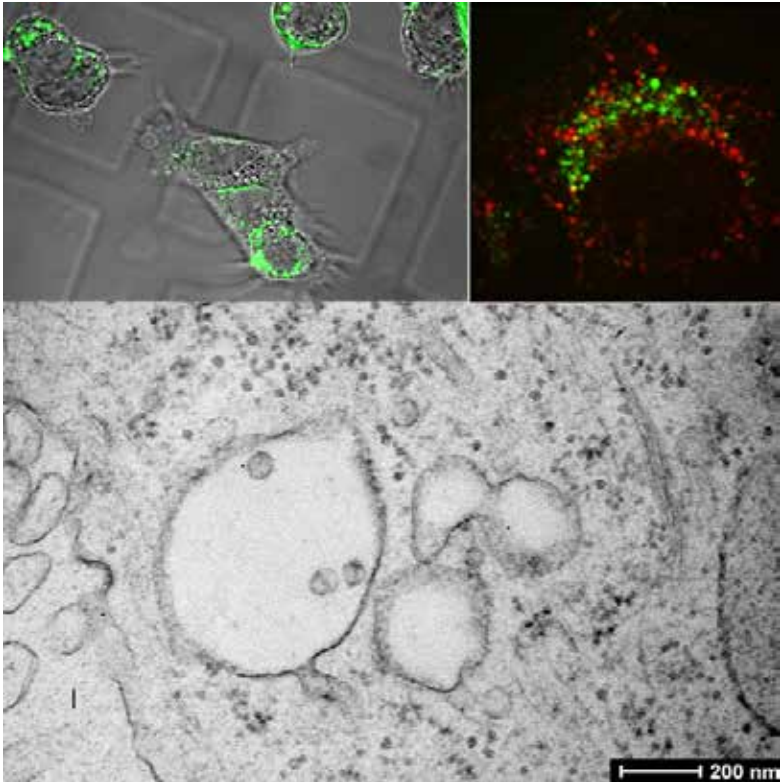
Image sequence showing different experimental stages of a CLEM experiment. Top left; HeLa cells growing on finder glass coverslips. The cells have internalised EGF coupled to Quantum dots 565. Top right; HeLa cells at late stages of internalisation with EGF-Alexa594 and Transferrin-Alexa488. Bottom; Electron Microscopy image of a HeLa cell internalised with EGF-Alexa488-10nm gold and Transferrin-Alexa594-5nm gold (see also Brown et al., 2012 Methods in Cell Biology, Volume 111: 175-201). Image courtesy of: Edward Brown and Paul Verkade, Wolfson Bioimaging Facility, University of Bristol.

remain with the cells as they move from LM to EM. Using this approach, the position of the cells can be determined using the grid numbers as a reference.

Other ways of highlighting an area of interest include carefully removing the cells surrounding the area or scratching an outline of the area with a diamond pen. To help in this regard, some systems include an objective holder with a diamond instead of a lens; this holder is lowered gently onto the cell culture dish to circle the area of interest, which can then be further processed for EM analysis.

Image alignment can also be greatly simplified by adding reference markers, commonly referred to as fiducial markers. With this method, developed by John Briggs from the European Molecular Biology Laboratory (EMBL) in Germany, the region of interest can easily be identified in relation to the position of the marker. To test this approach, the team successfully identified HIV particles bound to cells, and imaged in detail the scission event during endocytosis in budding yeast.

A significant constraint in CLEM is the time consuming and laborious process of physically transferring samples from the light microscope to the electron microscope for analysis. It's easy to see why researchers are working towards a completely automated CLEM system to eliminate the need to rely on two separate microscopes, thereby greatly increasing the speed of the technique.

The biggest challenge in transferring samples is caused by the fact that the sample preparation requirements of LM and EM are very different. For example, visualization of live cells requires an aqueous solution to keep cells alive and probes fluorescent, while the cells need to be plunged into cryo-fixative before EM examination.

Recently, several automated methods for switching samples between LM and EM have been proposed. One of them, dubbed the

first truly correlative scanning electron and optical microscope, came from Ian Morrison's team at the University of York, UK. In this system, an electron-permeable silicon nitride membrane allows the electron beam to reach the sample from underneath; while the light microscope is positioned above the culture dish. The cells can be observed live with LM and then fixed and stained *in situ* for EM analysis, greatly reducing sample preparation time.

A second example was proposed by Jacob Hoogenboom from Delft University of Technology in the Netherlands and involved placing an optical excitation and detection unit inside a scanning electron microscope. This simple, yet powerful solution allows quick image identification in LM mode combined with rapid location via EM, automatically matching corresponding images and taking hours off of the manual process.

CASE STUDY 1

To study the incredibly dynamic nature of the digestive vesicles known as lysosomes, Judith Klumperman and her team, based at the Cell Microscopy Centre at the University Medical Centre Utrecht in the Netherlands, rely on many microscopy techniques, including CLEM. Historically, the group relied on EM, but soon realised CLEM's potential in their work. 'We can have separate information of the LM and separate information of the EM, and that is already very valuable, but [with CLEM] you can integrate it down to the same organelle,' says Klumperman. 'Our separate data becomes so much more.'

The group has already identified several ubiquitin ligases involved in the destruction of specific proteins in the lysosome, and recently added TRIAD1 to the list. Using CLEM, they've shown that restricting the activity of this new ubiquitin ligase affects the function of lysosomes and causes proteins tagged for destruction to end up accumulating inside misshapen endosomes. Their approach involved preparing fluorescently-tagged ultrathin cryo-sections to identify cells of interest. The selected sections were then gold-labelled and transferred to the EM for detailed analysis of protein localization and ultrastructural information.

Klumperman believes CLEM will become a key microscopy technique in the future. 'For me, CLEM has really bridged the gap between LM and EM,' she says, '[and] more and more people that do advanced LM also want to use EM to add the final part of information that can only be obtained by EM.'

*[Biology Open, 2012, **1**, 607–614 (DOI: 10.1242/bio.2012778)]*

CASE STUDY 2
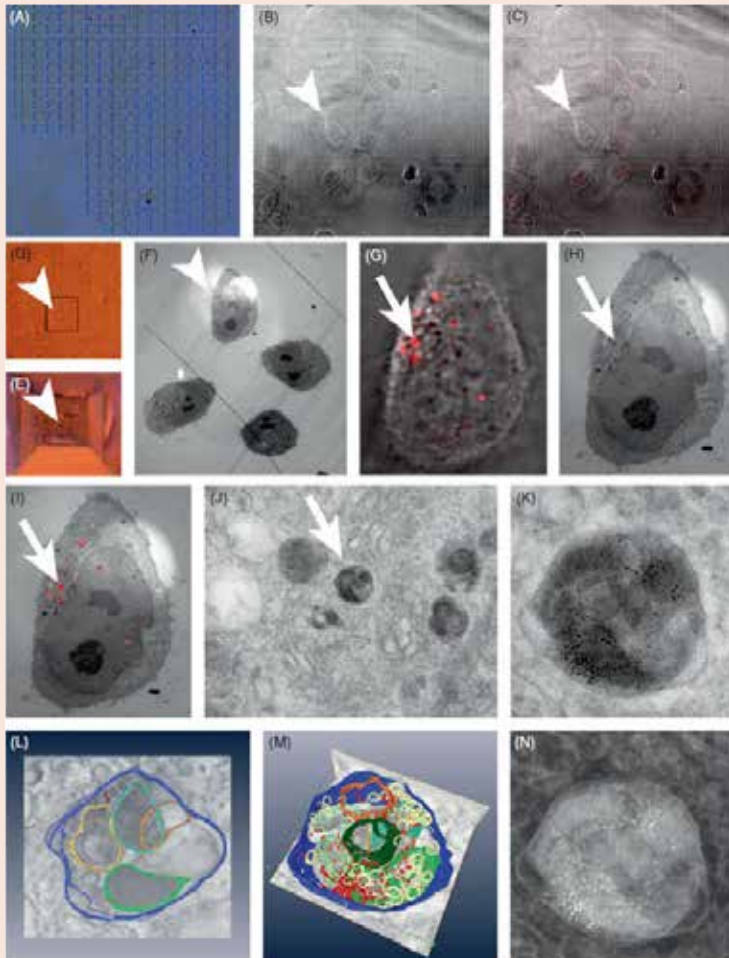
A simple CLEM experiment including 3-D reconstruction by electron tomography. Example images of a CLEM experiment

(A) The finder pattern of the embossed coverslip. (B) DIC image of HeLa cells grown on the embossed coverslip. Arrowhead points to the cell of interest (images B–F). (C) Overlay of B and a fluorescence image of the cells that have internalized EGF-biotin coupled to streptavidin-QD 655. (D) Stereomicroscopic image of the region of the cells of interest after chemical fixation and embedding in the Epon resin. (E) As D after trimming the block face. (F) EM overview image highlighting the area containing the cell of interest. (G) Zoomed image of the overlay of the DIC and fluorescence of the cell of interest. Arrow points to the organelle containing EGF-biotin coupled to streptavidin-QD 655 (image G–J). (H) EM image of the same region as G. (I) Overlay of the fluorescence image and the electron micrograph of the same region as G. (J) Electron micrograph of the subcellular area containing the organelle of interest. (K) Standard high-magnification bright field EM image of the organelle of interest. (L) Screen shot of the modeling part of an electron tomography experiment where structures of interest have to be manually segmented. (M) Screen shot of the modeling of the structure of interest. (N) HAADF image of the same region as K.

Jan R.T. van Weering et al. *Intracellular Membrane Traffic at High Resolution*

The research group led by Paul Verkade at Bristol University, UK, is well-known within the CLEM community. Their research focuses on understanding and imaging intracellular transport processes, mainly using correlative microscopy techniques they have developed themselves.

One of the ways cells regulate intracellular trafficking is by forming membrane tubules. However, tubule numbers are limited and at any given point there may only be a few present in the cell. Verkade explains that CLEM is the best option for studying these tubules, as they are sparse events that can be imaged easier with CLEM than with EM on its own.

Verkade adds that he prefers to use cryo-CLEM rather than standard chemical fixation. 'I am working on membrane tubules inside cells, but these fall apart on chemical fixation so cryo-fixation is essential,' he explains. 'Our main technique involves using live cell imaging, cryo-fixation with a special device (Leica EMPACT2 + RTS), freeze substitution, and electron tomography.'

Using this approach, developed in 2008, they were able to capture specific segregation and fusion events between two cellular organelles for the first time. In their latest work, the group tweaked the original method and revealed a 3D tomographic model of the endosome, which is a central component of the membrane transport pathway. From these reconstructed images, it is possible to see that compartments that appeared distinct are actually part of the same complex structure.

'Almost any kind of research that uses light and/or electron microscopy might benefit by using a CLEM approach,' concludes Verkade. 'There is a drive for integrated instruments but that so far has always resulted in compromises and limited use. A biosynthetic particulate CLEM probe is for me the Holy Grail for CLEM.'

CASE STUDY 3

Although the process of cell division has been investigated in all its minute details, the mechanism regulating its final step is still poorly understood. In a study to clarify which events lead to complete division (known as abscission), a research team from the Technical University of Dresden in Germany used CLEM to image live HeLa cells expressing GFP-tagged tubulin, followed by fixation before serial thin-section EM. In this case, the use of CLEM, in combination with super-resolution microscopy and electron tomography, permitted researchers to identify a new protein involved in the assembly of a specialised structure that mediates abscission.

'The combination of light and electron microscopy is very powerful as it provides data on dynamics (eg how do organelles or vesicles move around in the cell, how fast are structures assembled or disassembled) with high resolution structural data, giving a detailed insight into structure, arrangement and sizes of the cellular world,' says team leader Thomas Müller-Reichert. 'If we would not be able to do this, we would probably have to prepare many more samples to find one embryo or cell that is in the right stage. CLEM simply helps to increase the sample size.'

The team is not new to the technique and has published extensively, using CLEM to study how the microtubule cytoskeleton is modulated during meiosis and mitosis in the early embryo of C. elegans. Despite the obvious advantages, Mueller-Reichert also highlights certain limitations: 'There is still no perfect technique today, and one has to choose carefully the CLEM technique to use. We still have to compromise on things, such as structural preservation vs. preservation of fluorescent signal or the ability for a precise localization of the structure of interest.'

PROBLEMS AND SOLUTIONS

Although being able to locate a cellular structure or process with LM and then zoom in on it with EM is the central advantage of CLEM, actually locating the region of interest when shifting from LM to EM observations is fraught with difficulty. This is because the mechanisms of contrast in both microscopes are widely different, and even the same image can be unrecognisable between the two platforms.

To overcome this problem, specialised sample holders with grids or fiducial markers are essential (see image alignment). However, using these grids is not always straightforward and they may require considerable patience to set up. In addition, the presence of a grid can interfere with imaging, especially if the cell of interest is near a grid bar. The ability to keep track of the region of interest also depends on these grids remaining in a fixed position throughout the freezing and embedding process. If they move, it may not be possible to relocate the same cell again and the entire sample can be lost.

These are common approaches to finding the original region of interest, but even if the process is accurate, the searching operation is still likely to be time-consuming and tedious. One way to avoid this is to use a confocal microscope to select sections of interest, before moving on to EM examinations. This is particularly true if looking for a small or rare organelles like endosomes, which are only about 500nm in diameter. This could mean, for example, that out of 20 sections done in the selected cell, only two or three may be of interest.

However, even before scientists get the chance to analyse regions of interest, there is the more basic limitation of how to grow

the desired cells. For in order to detect fast moving cellular processes, such as membrane trafficking or cell division, the cells must be visualised in the light microscope in a carrier already appropriate for subsequent freezing. Otherwise researchers run the risk of completely missing their desired target, as transferring cells may take too long.

If using HPF, cells are grown in a sealable cavity known as a specimen carrier. The carrier used must have a high thermal conductivity to ensure that it does not slow down the rate of heat loss during freezing. Most common carriers are made from copper with a gold coating or from aluminium. The problem is that copper is extremely toxic for some cells and in some cases the copper ions can pass through the gold layer, while aluminium will oxidize when using osmium and so this will also damage the sample when processed. In this case, the only solution is to try and try again.

Another advantage of HPF is that it avoids destruction of cellular structures, which is a common consequence of chemical fixation. Breakdown of organelles results in a series of small vesicles that may cause great difficulties when correlating LM and EM images, not to mention misinterpreting the results. It is important to confirm that the structure observed under EM overlays with images obtained from LM. To avoid artefacts, the LM probe must produce the same pattern as the EM probe.

The eternal problem with CLEM is the lack of compatibility between probes and techniques. For example, some probes, including ReAsh or miniSOG, are not compatible with HPF; in contrast, GFP or Qdots fluorescence may be salvaged if freeze substitution is done afterwards. When setting up CLEM, it is vital that researchers are aware of possible combinations relevant to their research,

but the best solution may only be discovered by trial and error. For example, options to consider may include trying different probes or altering the cocktail of chemicals used during chemical or freeze substitution, including eliminating glutaraldehyde and reducing osmium tetroxide. The goal is to reach the best compromise between structure and fluorescence.

Ultimately, despite all the progress in automating and improving certain steps in the CLEM approach, one of its main limitations remains the fact that it is a complex and time-consuming technique, heavily reliant on staff with specific CLEM expertise. Each experiment may take several days to run, from sample preparation to EM examination and data analysis; and with so much handling, samples may be prone to contamination or damage. This severely limits the amount of data that can be extracted from a CLEM experiment and puts a strong emphasis on the success of sample preparation and staining procedures.

WHAT'S NEXT?

Forecasting the future of such a rapidly evolving subject is difficult, but there are indications of how CLEM may develop. Interestingly, over the past two decades, light microscopy has been revitalised, particularly with recent advances in super-resolution techniques and new fluorescent proteins. These new super-resolution techniques allow fluorescence microscopy to bypass the diffraction limit, which has always restricted the resolution of light microscopy to around 250nm, achieving resolutions below 50nm.

As a consequence, some had questioned whether there was still a need for the electron microscope. Instead, what's happening is that more and more research groups utilizing these advanced microscopy technologies are finding themselves faced with questions that can only be answered by EM.

This means the most likely future for correlative microscopy involves more collaboration, with CLEM increasingly integrated with and complemented by these new super-resolution microscopy techniques. Indeed, super-resolution microscopy is probably easier to integrate with EM than conventional light microscopy, as their resolutions are more comparable, allowing proteins to be localized with much less ambiguity.

Some of the first demonstrations of this kind of cooperation have come from groups led by Harald Hess, now based at Howard Hughes Medical Institute's Janelia Farm Research Campus in Virginia, USA, and Erik Jorgensen at the University of Utah, USA. These research groups chose photoactivatable localisation microscopy (PALM) or stimulated emission depletion microscopy (STED) to visualise cellular structures superimposed with EM images. Other groups are attempting further combinations and, as integration

develops, the complexity of the systems used will most likely increase, perhaps even allowing investigations of living cells in space and time (4D).

A glimpse of what's to come in the future comes from a recent study by a team led by Thomas Cremer at the Ludwig-Maximilians University in Munich, Germany. To explore the features of the nucleus in HeLa cells, they used spinning disk laser scanning microscopy (SDLSM), followed by 3D confocal laser scanning microscopy (CLSM), 3D structured illumination microscopy (3D-SIM) and transmission electron microscopy (TEM).

In addition, the scope of correlative techniques will broaden significantly, to include other complex microscopy techniques, such as fluorescence correlation spectroscopy (FCS), raster-scan image correlation spectroscopy (RICS) and total internal reflection fluorescence (TIRF). It is now obvious that no single microscopy platform is going to triumph over the others, singlehandedly providing a comprehensive understanding of cellular mechanisms. The best way to move forward is to realize that every technique can make important contributions to correlative efforts, with each helping to overcome the others' limitations.

The trick is to develop accurate ways to compensate for different resolutions and distortions. In addition to hardware improvements, this will require software to be greatly upgraded, in particular to enhance three-dimensional analysis after imaging, as well as developing automated pattern recognition software to reduce user input.

An important limitation at the moment is the fact that there is currently no single marker that is simultaneously visible by LM (fluorescent tag) and EM (electron-dense tag), as well as being

HPF-friendly. This means CLEM relies on complicated sample preparation procedures with dual labelling, which is time-consuming and complex.

Researchers are looking to develop a single probe for CLEM, but they're not sure yet whether the best approach is going to be genetically-encoded or affinity-based. Either way, a single probe would simplify sample preparation, and allow the labelled specimen to be clearly detected and localised with increased precision. Such probes could become widely available for a wide range of fields, from basic cell biology to biomedical and pharmaceutical research. One of the current ideas in development involves a novel light microscopy technique called four wave Mixing microscopy (FWMM) and the use of gold as the only probe for both LM and EM, following HPF and freeze substitution.

From a practical point of view, when developing CLEM for the first time, many research labs struggle for months trying to reconcile incompatible equipment, which greatly limits their chances of success and can be extremely expensive. To avoid this steep learning curve, a significant step forward would be to develop commercially available 'all-in-one' solutions for CLEM, thereby opening up this technique to research scientists who may have limited microscopy experience.

Thinking long-term, there are a few problems that have yet to find a solution. As an example, capturing events that may occur within milliseconds is still a challenging endeavour. Nowadays, it is possible to image live cells and, after detecting an important event, cryo-fix within less than five seconds to freeze the process. However, events that occur much faster than this, such as synaptic vesicle fusion, cannot be captured by CLEM. Future developments

may also include reducing the distance between the sample and the objective lens, which can be as high as 180μm and so is at the far end of working distances for high magnification objectives.

If all these developments come to pass, CLEM will truly become a key technique for deciphering the complexity of cellular mechanisms. By offering a way to flow seamlessly between light and electron microscopy, it will provide biologists with the opportunity to zoom in and out of individual cells at will.

FURTHER INFORMATION

CLEM section of Leica website (http://www.leica-microsystems.com/science-lab/tag/tags/clem/show/Tag/)

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