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Review Article

A Review of Correlative Light and Electron Microscopy (CLEM) Methods, Markers, and Instrument Set Ups to Study Infectious Disease

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Abstract

Correlative light and electron microscopy (CLEM) is an effective technique used to study biological samples. Signal-specific indicators observable under light microscopy (LM) allow scientists to locate areas of interest for high resolution ultra-structure observation under the electron microscope (EM). Recent developments in method have provided breakthroughs creating an effective, direct, and accurate research diagnostic tool for functionally related structural biological studies. CLEM is particularly useful in infectious disease research where there is need to study, at nanoscale, objects of interest which are commonly part of rare transient events or afflict a particular cell among a majority of unaffected cells. In this review, we discuss several CLEM methods, summarize currently available fluorescent markers, and discuss CLEM instrumentation setups for novel approaches to imaging cellular events in infectious disease.

Keywords: *CLEM; Infectious Disease; Fluorescent Markers; Ultrastructure*

Introduction

Imaging is an important diagnostic and research tool in infectious disease [1-3]. Attempts to see small pathogens and organisms started in the 17th century with Antoni van Leeuwenhoek's light microscope (LM) [4]. The electron microscope (EM) was used to observe viruses for the first time in 1937. Currently, with cryo-EM methods, angstrom resolution imaging can be achieved revealing virus ultrastructural detail at the molecular level [5]. Since the first reported observation of fluoresce in 1845, fluorescence microscopy has significantly evolved and contributed to many major discoveries [6]. In recent decades, the development of super-resolution microscopy techniques such as photo-activated localization microscopy (PALM) [7], stimulated emission depletion (STED) microscopy [8], stimulated emission microscopy (SIM) [9], and stochastic optical reconstruction microscopy (STORM) [10], achieved resolution beyond the diffraction limit of 200 nm. Super-resolution light microscopy imaging techniques have been implemented with success in the study of infectious diseases [2]. Although these techniques achieved unprecedented resolutions below 50nm, resolution below 5 nanometers, thus far only achievable by EM, are still essential to study viruses. EM provides spatial resolution and context that complements LM; therefore, EM imaging techniques combined with using correlative methods with LM have an important role in infectious disease research.

There are drastic differences in the LM and EM imaging techniques and sample preparation protocols. These differences create advantages and limitations that can compensate for each other. LM has a large field of view that provides the flexibility of live cell imaging, and is suitable to study cellular events and dynamic processes with fluorescent markers. EM can identify ultrastructure information, revealing cellular relationships and interactions which provide spatial resolution and context for the object of interest. Spatial resolution and context is enhanced by EM because staining of bulk membranes and proteins delineates organelles and other structures surrounding the object of interest. This cannot be done with fluorescent LM as it only detects the object of interest labeled with the fluorescent marker.

Combining LM and EM imaging systems is a valuable diagnostic and research tool for nanoscale study of pathogens and their interaction with the host cell in infectious disease.

Various approaches to correlation of microscopy techniques existed since the early 60's [11,12]. Nearly all EM studies have some degree of correlation to light microscopy, but the level and accuracy of this correlation has improved over the years. For many decades, the correlative light and electron microscopy (CLEM) studies used an approach where samples went through different preparation: formalin fixation and paraffin embedding for LM versus aldehyde fixation, osmium tetroxide staining, and epoxy resin embedding for EM [13]. The sample was investigated using both types of microscopy but correlation to the cellular level was impossible. Recently, several techniques and equipment have been developed that improve the accuracy of CLEM correlations by enabling imaging of the exact cellular location with both modalities. These improvements allow for correlation to the single cell as well as the inclusion of time-resolved images enhancing the study of virus replication and production [14-17]. In this review, we focus on the use of various CLEM methods, fluorescent tags used in CLEM, and instrument setups beneficial to research in infectious disease. We only discuss methods involving thin sections on EM grids and will not review cryo-CLEM methods [18,19] that observe vitrified samples under cryo-conditions.

CLEM Methods and Strategy for Sample Processing

There are three unique CLEM sample processing methods important for studying infectious diseases (Figure 1): (1) pre-embedding CLEM where the sample is imaged with LM before embedding into plastic resin for EM observation, (2) post-embedding CLEM where the sample is imaged with LM after embedding into plastic resin for EM observation, and (3) Tokuyasu CLEM, where the sample is prepared by cryofixation and LM is done before EM observation. All three CLEM sample preparation methods use fluorescent markers to identify or pre-select cellular targets exhibiting events such as viral entry, replication, and shedding. Pre-identification through LM provides focus for EM investigation, an efficient approach to the "needle in a haystack" challenge often associated with EM. Each method has advantages and disadvantages which should be carefully considered during study design.

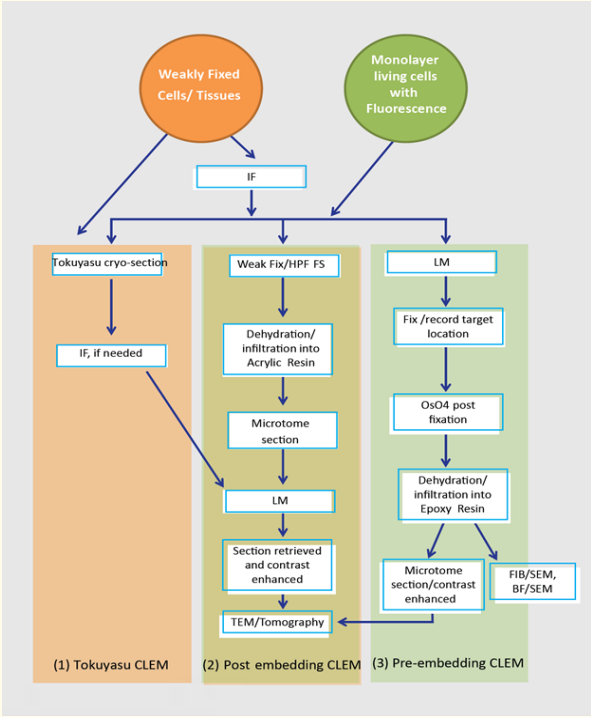

Figure 1: *CLEM methods overview.*

Pre-embedding CLEM

Due to live cell imaging capability, recording cellular time points, and excellent preservation of morphology, pre-embedding CLEM is the most practical CLEM method in infectious disease studies. It is especially useful when studying viral replication and the dynamics of cellular interaction with viruses [14,15,20]. Fluorescent markers are used as indicators for objects, time points, or events under LM. Once the LM target and location is identified, the sample is immediately processed for EM. Fixative is added as soon as the cellular event is observed under LM. Cell location is identified using a MatTek®gridded petridish [21] or markings on the glass coverslip [22]. Samples then go through conventional EM preparation and are embedded into epoxy resin. When incorporated with EM tomography this method is considered 4D microscopy-3D structure plus time [21].

The most significant advantage to using pre-embedding CLEM for infectious disease research is its ability to capture specific cellular events during a dynamic cellular process and proceed with methods ideal for ultrastructural detail under EM. When this method is combined with high pressure freeze (HPF) and freeze substitution (FS) sample preparation [23,24] this method provides EM morphology preservation superior to any other method we discuss in this review. Criticisms of this method include potential changes in the specimen due to the small time delay in fixation and non-correlation in the z-axis between LM and EM images [25]. For most instances this time delay in fixation is considered negligible and insignificant, but in the event of very transient events this delay may be consequential. Picking the closest approximate slice from the confocal image stack to match the EM image [26] or using 3D volume EM reconstruction by focused ion beam (FIB)-SEM and serial block face (SBF)-SEM to match the confocal image stack [27] helps correct z-axis correlation between LM and EM systems.

Pre-embedding CLEM can only be applied to monolayer cells or very thin samples visible under LM since this is used to mark the location for EM investigation. This method's flexibility, practicality, and superior EM morphology make pre-embedding CLEM a great option for most infectious disease studies. The flexibility of this method comes from its ability to study dynamic cellular interactions and is practical because any type of light microscope and a wide variety of fluorescence markers can be used, and special equipment or skill sets are not required.

Post embedding CLEM

In post-embedding CLEM, LM is performed after embedding into resin for EM. In this method, samples tagged with fluorescence markers are fixed, dehydrated and infiltrated into resin. Most post-embedding CLEM methods use acrylic resins such as Lowicryl HM20 [25,28], LR White [26], and Glycol Methacrylate (GMA) [29,30] because they are hydrophilic and favorably interact with fluorophores. Although Epon epoxy resin is the best embedding material for morphologic preservation, its incompatibility with water results in complete fluorescence quenching. Etching and antigen retrieval through heat enables IF staining on Epon sections for potential CLEM applications [31]; however, this is technically challenging and depends heavily on the behavior of individual antibodies. Post embedding CLEM provides the distinct advantage of acquiring LM and EM images on the same ultrathin section without additional manipulation between imaging modalities, resulting in the most exact correlation possible. Correlation can be accurate down to the molecular level in some cases [29].

The advantage of post-embedding CLEM is easy handling of the plastic sections and their stability under the electron beam. In infectious disease studies, if the fluorophore tagged target is big enough, such as a bacteria or large virus, consecutive sections can be imaged using different imaging systems such as LM, super-resolution fluorescence microscopy, TEM and SEM [29]. In the consecutive-section approach, adjacent ultra-thin sections are imaged using different microscopes. Although this approach does not result in correlation to the single molecular level, it provides information for the same target from adjacent sections with different observation systems. There is no need to switch samples among systems, so it is much faster and easier than the same section approach. The same section approach [29] is desired in infectious disease research since many viral targets are smaller than 80 nm, the average thickness of an ultra-thin section. Future developments simplifying the transferring of sections between different imaging systems will also enable successful CLEM imaging of serial sections to build up 3D correlation for studying larger targets.

The most consequential limitation for post embedding CLEM is the need to avoid heavy metals during sample preparation to prevent the quenching of fluorescence. The lack of heavy metals in sample preparation results in low contrast and extensive extraction from tissue dehydration [25,26]. HPF-FS can overcome extraction issues and greatly improve morphologic preservation [28,32].

Post embedding CLEM can be applied to monolayer cells, cell pellets and tissues. The most useful feature for post embedding CLEM is flexibility due to ease of section handling – either with consecutive or same-section approaches. This is the best method for precise correlation of CLEM, especially to study a larger target using serial sections for 3D volume regeneration.

Tokuyasu CLEM

The Tokuyasu method was originally developed for the purposes of providing improved immunogold labeling of ultrathin cryosections, but it also has been adapted for use in CLEM protocols [33,34]. Instead of immuno-gold, CLEM procedures apply immunofluorescent (IF) labeling to cryosections that are subsequently captured with LM and EM on the same section [35].

There are several advantages of Tokuyasu CLEM. First, it retains antigenicity better than either of the other methods. This increases labeling efficiency due to the openly accessible antigens in a non-resin environment. The unique ability to preserve antigenicity in this method may provide a good last resort when using sensitive antibodies that fail to stain using other methods. Preserved antigenicity also enables both IF and immuno-gold labeling for more precise correlation [36]. Secondly, Tokuyasu is the fastest CLEM method. Processing only takes a couple of hours because shorter infiltration periods are required when processing into plastic resin after cryo fixation [34].

Cryosectioning is a difficult technique and creates challenging limitations in Tokuyasu CLEM. Cryosectioning requires a small surface sample size, typically less than 0.2 mm X 0.2 mm, to obtain high quality cryosections [37]. This leaves only a small area to be imaged. Additionally, excellent cryosectioning skills are rare and difficult to acquire. If cryosectioning expertise is not available this technique is not recommended.

Tokuyasu CLEM works well with cell pellets and tissues; additionally, recent clever modifications of this method has enabled it be applied to studies of living cell monolayers [38,39]. This method combines live-cell fluorescent imaging and immunogold labeling of ultrathin cryosections to study the dynamics of membrane-bound organelles [40,41]. Overall, Tokuyasu CLEM is the most beneficial for samples that require sensitive protocols for immuno-labeling [42].

CLEM Fluorescence Markers

Fluorescent markers have improved light microscopy by offering high sensitivity, improved spatial resolution, multiple tagging, and the ability to image live cells [43]. With the development of CLEM, scientists have found ways to view markers with both light and electron microscopes. While dual observation of the signal with both light and electron beams provides the most accurate correlation, sufficient accuracy for a study may be achieved by combining the data from separate images using overlays. Often, fluorescent images are taken on pre-embedded samples mounted on a gridded system, and the gridded system provides the location of objects of interest for EM imaging [14-16,21,26,44]. Here we discuss fluorescent proteins, synthetic fluorophores and particles, and diaminobenzidine photooxidation for the use of correlating light microscopy signals to EM observations (Table 1).

Category	Common Markers used in CLEM	Advantage	Disadvantage
Fluorescence Protein	• Green Fluorescent Proteins (GFP) and the variants • Yellow Fluorescent Proteins (YFP) and the variants • Red Fluorescent Proteins (RFP) and the variants • Blue and Cyan Fluorescent Proteins (YFP) and the variants • Eos Fluorescent Protein and the mEos variants.	• Endogenous expression for live cell imaging • Eos/mEos for Photo-activated localization Microscopy (PALM) and EM correlation studies.	• Relatively low brightness • Large size >25 KD possibly perturbs the target protein
Synthetic Fluorophore and Particles	• Commercially available fluorophore labeled antibodies and peptides • Selective labeling of fusion proteins such as FlAsh and ReAsh • Quantum dots(Q-dots)	• More colors and wavelength ranges • Brightness • Q-dots are visible under both LM and EM	• Need chemical procedure to deliver
Photo-Oxidation using DAB	• Lucifer Yellow • HRP/APEX conjugates or co-expression • BODIPY conjugates • Eosin conjugated reagents • ReAsh • MiniSOG	• Directly visible under both LM and EM • Provide high quality EM morphology preservation	• Less sensible • technically challenging to have good staining and accurate distribution

Table 1: *Comparisons of current common markers in CLEM.*

Fluorescent Proteins

Fluorescent proteins (FPs) are a diverse family of structurally homologous chemiluminescent proteins that self-sufficiently form a visible wavelength chromophore. Fluorescent proteins commonly used include green fluorescence protein (GFP) from jellyfish [45], red fluorescence protein (RFP) from corals [46,47], and the coral-derived green-to-red photoconvertible fluorescent protein EosFP [48]. Additionally, through mutagenesis and protein engineering, FP variants featuring fluorescence emission spanning much wider regions of the visible spectrum have been developed including blue, cyan, green, yellow, orange, red and far-red [49-52]. Dehydration and heavy metal staining steps in routine EM processing quench fluorescence or destroy antigenicity, providing great challenges in maintaining fluorescent signal in samples when FPs are used [25,26,30,43,53]. Techniques that maintain fluorescence require a compromise during EM processing including changes such as decreased concentrations of uranyl acetate (UA) and osmium tetroxide (OsO4) during fixation, and use of hydrophilic resins. Protocols have been developed which maintain GFP fluorescence through pre-embedding [54] and post-embedding [26,28] in various hydrophilic resins. Other FPs that have been successfully used for post-embedding staining in hydrophilic resins including RFP [26,28] YFP [30] and mEos [29,30]. Most notably, an example of a fluorescent protein that withstands EM processing is mEos4. It has been shown to handle standard EM OsO_4 fixation concentrations of 1% [29].

FPs are valuable due to the endogenous expression of the proteins making them compatible with live cell imaging. However, since FPs are relatively large, with a typical size of 2 - 5 nm and a mass of at least 25KD, there are concerns that the size of the tag and oligomer formation may affect the function of the target protein and introduce artifact [55].

Synthetic Fluorophores and Fluorescence particles

Synthetic fluorophores are fluorescent chemical compounds that re-emit light upon excitation. These compounds are popular in CLEM because they are hardier than FPs and can better survive EM processing, provide more colors and wavelength ranges, and achieve higher resolution [25]. As opposed to FPs, delivery of synthetic fluorophores into live cells requires invasive techniques such as microinjection, site-specific incorporation of unnatural fluorescent amino acids, or selective labeling of fusion proteins [55]. For example, FlAsh and ReAsh use a fused tetracystein tag which reacts with biarsenical fluorophores to form highly stable fluorescent complexes [21,56-58]. Other CLEM protocols have been developed for pre-embedding staining with Alexa Flour, tetramethylrhodamine (TMR), and silicon-containing rhodamine derivative Sir-carboxyl (SiR) [25] in live cells. Immunofluorescent labeling using synthetic fluorophores in fixed cells and tissues is easily done compared to live cell imaging. There is a large selection of commercially available fluorophore-labeled antibodies and peptides that have been developed for this purpose [59,60].

A relatively new technology in synthetic fluorescent particles is quantum dots (Q-dots). These particles are small semiconductor nanocrystals that provide high fluorescent yields and resist photo bleaching [53,61,62]. They contain a dense metal core visible under EM which makes fluorescent preservation non-essential [53,61,62]. Since it is unnecessary to preserve fluorescence, ideal EM processing techniques and resins can be used for better ultrastructural morphology preservation [53,61-63]. Q-dots also lend themselves well to multiple labeling for CLEM [53,61,62]. They can be distinguished by color under LM and by shape and size of their metal core under EM. Currently, there has been successful use and differentiation of 3 different Q-dots tags [53]. Of note, the fluorescence of Q-dots is destroyed by OsO4. If post embedded fluorescent signal is desired for same section imaging then some compromise in EM processing may be required [53,61].

Diaminobenzidine (DAB) Photooxidation

There are several fluorophores that can create an oxidizing reaction with DAB, resulting in a localized osmophilic precipitate visible with EM [64,65]. Examples include Lucifer yellow [66], Horseradish Peroxidase (HRP) conjugated IF antibodies [67] or co-expression with FP [68], boron-dipyrromethene (BODIPY) conjugates [69], eosin conjugated reagents [43], ReAsh [57], MiniSOG [70] and Click-EM [71]. The enzyme horseradish peroxidase (HRP) can oxidize DAB, but its staining capabilities are limited in the cytosol due to insufficient calcium levels for the reaction [72]. Ascorbate peroxidase (APEX) was developed to address this shortcoming of HRP and is able to react in all parts of the cell [72]. Similar to HRP, APEX cannot fluoresce, but can be used with various other fluorophores for LM visualization [70,72], however, APEX requires heme to react. Typically, endogenous heme is sufficient, but a heme rich media may be required for successful precipitation [72]. Multiple protocols have also been developed for the use of GFP, CFP, YFP, and BODIPY as photo oxidizers [73,74]. MiniSOG is another more recently developed small protein module which fluoresces and can photo convert DAB into a precipitate [70]. Its small size does not interfere with target proteins, which commonly occurs with larger tags. Additionally, it achieves a higher resolution than enzyme based methods because it does not require use of permeabilizing agents for penetration, and it can be used as a genetic tag [70,72,75]. The most recent advance in fluorescent DAB photooxidation includes a process known as Click-EM. Click-EM allows labelling of non-proteinaceous structures including nucleic acids, lipids, and glycans. In this method azide alkyne functionalized analogs of biomolecules are incorporated into cells and revealed by a reaction known as "click chemistry": Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC). This CuAAC reaction creates a label visible by fluorescence and subsequently by EM through photogeneration of singlet oxygen for DAB precipitation [71].

DAB photooxidation allows for conventional EM procedures and epoxy resins ideal for preservation of ultrastructural morphology because staining and the oxidation reaction occur prior to EM processing [70,72,75]. Some investigators consider photooxidation less desirable because it requires in-depth protocols and often results in uneven staining [29]. Other disadvantages when using photooxidation labels include non-specific mitochondrial staining [76], chemical interference with the oxidation reaction [43], and limited penetration [43,72]. In fixed and non-fixed cells mitochondria generate reactive oxygen species (ROS) [76] which photooxidize DAB, resulting in non-specific staining [43,75] but steps can be taken to minimize this [73]. Fluorescent anti-fade or brightening chemicals typically interfere with the oxidation reaction and should be avoided [43]. Penetration is typically limited without compromising stain localization. With the aid of permeabilizing agents the penetration of eosin to several microns has been shown, but these permeabilizing agents may cause organelle membrane damage allowing stain diffusion [43,72].

CLEM Instrumentation

Correlative methods enable visualization of the same structure utilizing the capabilities of typically separate, powerful microscopy platforms. Oftentimes, when applying CLEM in infectious disease research, the fluorescence image serves as a guide to find the corresponding cell during EM imaging. The biological question and available equipment determines which CLEM method should be applied.

CLEM can be performed with any dedicated electron and fluorescent microscopes; the same equipment as when each of the instrument imaging modalities are used independently. Integrated microscopes such as the FEI CorrSight™ and the iCorr™ were specially developed to perform both imaging modalities in a single instrument without movement of the sample. These microscopes require compromises in sample preparation to simultaneously fit the needs of both modalities. They are intended for same-section post-embedded imaging, and are not as useful when performing live cell imaging [59,77].

Previously, correlating the LM and EM images was very laborious. Various instruments and software packages have been developed to automate this task and improve the efficiency of obtaining images. Calibrated transfer shuttles orient the sample. An example is the Carl Zeiss Microscopy GmbH developed 'Shuttle & Find' system that uses coverslips with 3 fiducial markers to calibrate the positions of the images obtained in separate phases. After imaging with any dedicated light microscope, the coordinates of the object of interest are identified in relation to the fiducials on the coverslip. Software orients the sample based on the image. Examples include the Maps software (FEI Company) and the Atlas5 (Carl Zeiss Microscopy GmbH); either software package reads any type of image obtained by any light microscope. The Maps software is specially designed to correlate light and electron microscopy for the FEI CorrSight system, but also can be used with other systems. These software packages automatically orient the imported light microscope image using spatial landmarks such as fiducial markers or cell patterns [78-80].

The three most common CLEM instrument set ups that are of particular interest for infectious disease research include regular fluorescence or confocal imaging, live cell imaging, and super-resolution fluorescence imaging. All of these light microscopy techniques can be combined with any electron microscope imaging technique including TEM, EM Tomography, SEM, Focus Ion Beam (FIB)-SEM or block face-SEM (Figure 2).

Light Microscopy

	Resolution
Video	N/A
Confocal	200 nm
Super resolution	<50 nm

Room Temperature Electron Microscopy

	Resolution		
	X	Y	Z
Serial Sections TEM	<1nm	<1nm	30 nm
SEM	5 nm	5 nm	N/A
FIB-SEM	5 nm	5 nm	5 nm
Block-Face SEM	5 nm	5 nm	30 nm

Figure 2: *Comparison of maximum resolution of CLEM Instrumentation. Each of the light microscopy instrument types can be correlated with each type of electron microscopes on the right.*

Regular Fluorescence or Confocal Correlative EM

This family of LM requires a confocal or fluorescent microscope. Other equipment will depend on the method of sample preparation desired. For pre-embedding CLEM (LM performed prior to EM sample preparation) the equipment needs are the same as routine EM (ultramicrotome, sputter-deposition system, critical point dryer, and TEM or SEM microscope) and light microscopy (field inverted fluorescent microscope or confocal microscope system) performed independently. Additional instruments could include a sample holder with a grid or fiducial markers for assistance orienting the sample. An example of a gridded petri-dish is the MatTek® petri dish. This gridded sample dish allows the location of images obtained with LM to be oriented to the sample for electron microscopy imaging [21]. For example, the formation of the replication complexes in alphavirus infection was elucidated through CLEM correlation of images in MatTek® gridded petridishes [17,81]. The process of clathrin mediated endocytosis of Listeria monocytogenes was established by using a combination of pre-embedding fluorescence microscopy with TEM on HeLa cells grown on gridded coverslips [82].

Equipment required for samples prepared with post-embedding is similar as pre-embedded samples except this method requires a fluorescent marker that survives the harsh sample preparation conditions for EM imaging. Usually, EM procedures are modified to compromise for fluorescence preservation [26,29,32]. Tokayusa preparation requires specialized cryo-preparation equipment including a specialized ultramicrotome with a cryogen attachment and a cryo diamond knife.

Video-Correlative -EM

Observation of living cells under EM is impossible; however, a rare, transient event can be correlated with EM using in vivo fluorescence video microscopy [83]. Video CLEM can only be combined with the pre-embedding CLEM method discussed above. The location and dynamic of a target protein can be monitored by fusing a fluorescent protein with the target protein and allowing it to be expressed in the cell. GFP is commonly used for this purpose and can be fused with nearly any protein of interest [83]. Live cell, or video-CLEM, is a two-stage technique. In the first stage an imaging system capable of performing fast video microscopy (100ms/frame) is used to visualize the desired event. In the second stage the sample is fixed and processed for EM using the same equipment needed for routine EM imaging [83]. For example, pre-embedding video microscopy imaging of long periods (one frame every 10s) combined with SEM was used to visualize retrovirus budding and generate a comprehensive picture of retrovirus assembly and budding on the cell surface [16].

Correlative Super-Resolution LM and EM

Previously, light microscopy resolution beyond 200 nm was not possible due to the diffraction limit of light, but improvements in super-resolution microscopy has enabled these modalities to reach resolutions less than 50 nm and as low as 10 nm under ideal conditions.

Cellular structures previously only visible by EM can now be seen by LM. One super resolution microscopy technique called "localization microscopy" requires fluorophores, or small molecule dyes, intrinsically capable of photo-switching between activated and deactivated states. Alternative, random activation and deactivation creates an image of the specimen with nanometer localization accuracy. The current available localization microscopies are PALM [7,84] and STORM [10,85]. A CLEM set up of interferometric PALM (iPALM), combined with whole-cell mount pre-embedding SEM methods was used to study human immunodeficiency virus (HIV) endosomal sorting complexes required for transport (ESCRT) machinery at assembly sites [86].

Remarks and Perspectives

In this review, we have summarized CLEM methods, markers and instrument set-ups that can benefit infectious disease research. CLEM combines two individual imaging systems for direct observation of marker distribution correlated to the ultrastructural level for spatial details. CLEM has become more and more popular over the past 20 years due to advancements in marker development, sample preparation methods, and instrumentation. The resolution of CLEM correlation has not only improved within the x-y plane (2D), but has also significantly developed in the z plane (3D) with the combination of methods such as LM with EM tomography or FIB-SEM. CLEM is a powerful tool to study functionally-related structural changes and mechanisms at the cellular level. It is especially useful to study rare or unique cellular events in infectious disease. Correlating EM with advanced optical microscopy techniques overcomes the detection limit for small pathogens and reveals significant details essential to understanding their interaction with host cells.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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