Correlative Light and Electron Microscopy (CLEM) and its applications in infectious disease

Keith Koistinen, Candace Blancett, Mei Sun

Pathology Division, USAMRIID

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 method developments 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.

Introduction

Imaging is an important diagnostic and research tool in infectious disease [1-3]. Attempts to see small pathogens and organisms started in 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, and now 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 200nm. Super-resolution light microscopy imaging techniques have been implemented with success in the study of infectious diseases [2]. Although these techniques have achieved unprecedented resolutions below 50nm, resolution below 5 nanometers, thus far only achievable by EM, are still essential to study viruses. EM also provides the spatial resolution and context that complements LM. Therefore the imaging techniques available with EM combined through 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 have their own 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 that delineate 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 for the 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 the 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.

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 at the exact time point or moment that the cellular event is observed under LM. Cell location is identified using a MaTek 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 it 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, 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 section without additional manipulation between imaging modalities resulting in the most exact correlation possible; this accuracy of correlation can be accurate up to the molecular level in some cases [29].

The advantage of post-embedding CLEM is the 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 the adjacent ultra-thin section is imaged using different equipment. 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, easier when compared to 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 development in 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 dehydration of the tissue [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 handling of the sections – 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 as a good last resort when using sensitive antibodies that fail to stain using other methods. This also enables both IF and immuno-gold labeling for more precise correlation [36]. Secondly, this 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 which requires a small surface sample size to obtain high quality cryosections [37]; the technical difficulty and small sample size available for imaging both significant limitations in the Tokuyasu method. Typically samples must be less than 0.2 mm X 0.2 mm for good cryosectioning. Additionally, excellent cryosectioning skills are rare and difficult to acquire, so if cryosectioning expertise is not available this technique is not recommended.

Tokuyasu CLEM works well with cell pellets and tissues; additionally, recent clever modification enables Tokuyasu CLEM on 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 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 can be achieved if methods are used that combine the data from separate images (overlays). This overlaying of separate images is the most common method for correlative imaging. 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 (Figure 2).

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 (OsO_4) 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]. Mutants of fluorescent proteins which are able to better withstand EM processing are desirable but have not yet been developed or are in their earliest stages of development and implementation. Most notably, an example of a fluorescent protein that can withstand EM processing is mEos4 that has been shown to handle OsO_4 fixation by withstanding standard EM processing concentrations of 1% [29].

A great utility of FPs is the endogenous expression of the proteins for live cell imaging. The process of constructing and fusing FPs to almost any protein of interest inside cells is a technically mature and well characterized process[55]. However, since FPs are relatively large, with a typical size of 2-5 nm and a mass of at least 25KD, there are critiques and concerns that the size of the tag and oligomers formation may affect the function of the target protein and introduce artifact [56].

Synthetic Fluorophores and Fluorescence particles

Synthetic fluorophores are fluorescent chemical compounds that re-emit light upon light 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[56]. For example FlAsh and ReAsh use a fused tetracystein tag which reacts with biarsenical fluorophores to form highly stable fluorescent complexes. [21, 57-59]. 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. Contrary to living cells, the immunofluorescent labeling of cellular structures in fixed cells and tissues for using synthetic fluorophores is easily done. There is a large selection of commercially available fluorophore-labeled antibodies and peptides that have been developed for this purpose and an few examples include Alexa Fluor and TRITC [60, 61].

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, 62, 63]. They contain a dense metal core visible under EM which makes fluorescent preservation non-essential [53, 62, 63]. Since it is unnecessary to preserve fluorescence, ideal EM processing techniques and resins can be used for better ultrastructural morphology preservation [53, 62-64]. Q-dots also lend themselves well to multiple labeling for CLEM [53, 62, 63]. 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 OsO_4. If post embedded fluorescent signal is desired for same section imaging then some compromise in EM processing may be required [53, 62].

Diaminobenzidine (DAB) Photooxidation

DAB photooxidation works by the conversion of fluorescent signals into electron-dense precipitations. There are several fluorophores that can create an oxidizing reaction with DAB, resulting in a localized osmophilic precipitate visible with EM [65, 66]. Examples include Lucifer yellow[67], Horseradish Peroxidase (HRP) conjugated IF antibodies [68] or co-expression with FP[69], *boron-dipyrromethene* (BODIPY) conjugates[70], eosin conjugated reagents[43], ReAsh[58], and MiniSOG[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 [71, 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 photoxidizer that was developed very recently, and it is a small protein module which fluoresces and can photo convert DAB into a precipitate [71]. Its small size does not interference with target proteins as 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 [71, 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 chemistryCu(I)-catalyzed azide–alkyne cycloaddition (CuAAC). This CuAAC reaction creates a label that is visible by fluorescence and EM [76].

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 [71, 72, 75]. Some investigators consider photooxidation less desirable because it requires in-depth protocols and often results in uneven staining [29]. There are other disadvantages when using photooxidation labels and these include non-specific staining due to generation of reactive oxygen species (ROS) by mitochondria [77], interference of the oxidation reaction by fluorescent anti-fade or brightening chemicals [43], and limited penetration without permeabilizing agents. Mitochondria generate reactive oxygen species (ROS) [77] which photooxidize DAB resulting in nonspecific staining and this can occur even after fixation [43, 75], although steps can be taken to minimize this source of non-specific staining [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

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 applied 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, and are intended for same-section post-embedded imaging, and are not as useful when performing live cell imaging [60, 78].

Previously, correlating the LM and EM images was very laborious, but various instruments and software packages have been developed to automate these tasks and improve the efficiency of obtaining images. Calibrated transfer shuttles can orient the sample such as 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 images are stored in relation to the fiducials on the coverslip. Software can orient 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 [30,38, 57].

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 3).

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, 79]. 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 [80].

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 with 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[81]. 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 it is followed by 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. 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:. For example, pre-embedding video microscopy imaging of long periods (one frame every 10 s) 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 50nm and as low as 10nm under ideal conditions. Cellular structures previously only visible by EM can 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, 82] and STORM [10, 83]. 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[84].

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 and provides pinpoint correlation 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 not only improved within the x-y plane (2D), but has also significantly developed in the z plane as well (3D) with ability to combine methods such as LM with EM tomography or FIB-SEM. CLEM is a powerful tool to study functional-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 is very promising to overcome the detection limit for small pathogens and reveal significant understanding towards their interaction with host cells.

Acknowledgement and Disclaimer

This work is supported by US Army Medical Research Institute of Infectious Disease (USAMRIID) Pathology Division.

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

Figure 1 CLEM Methods overview

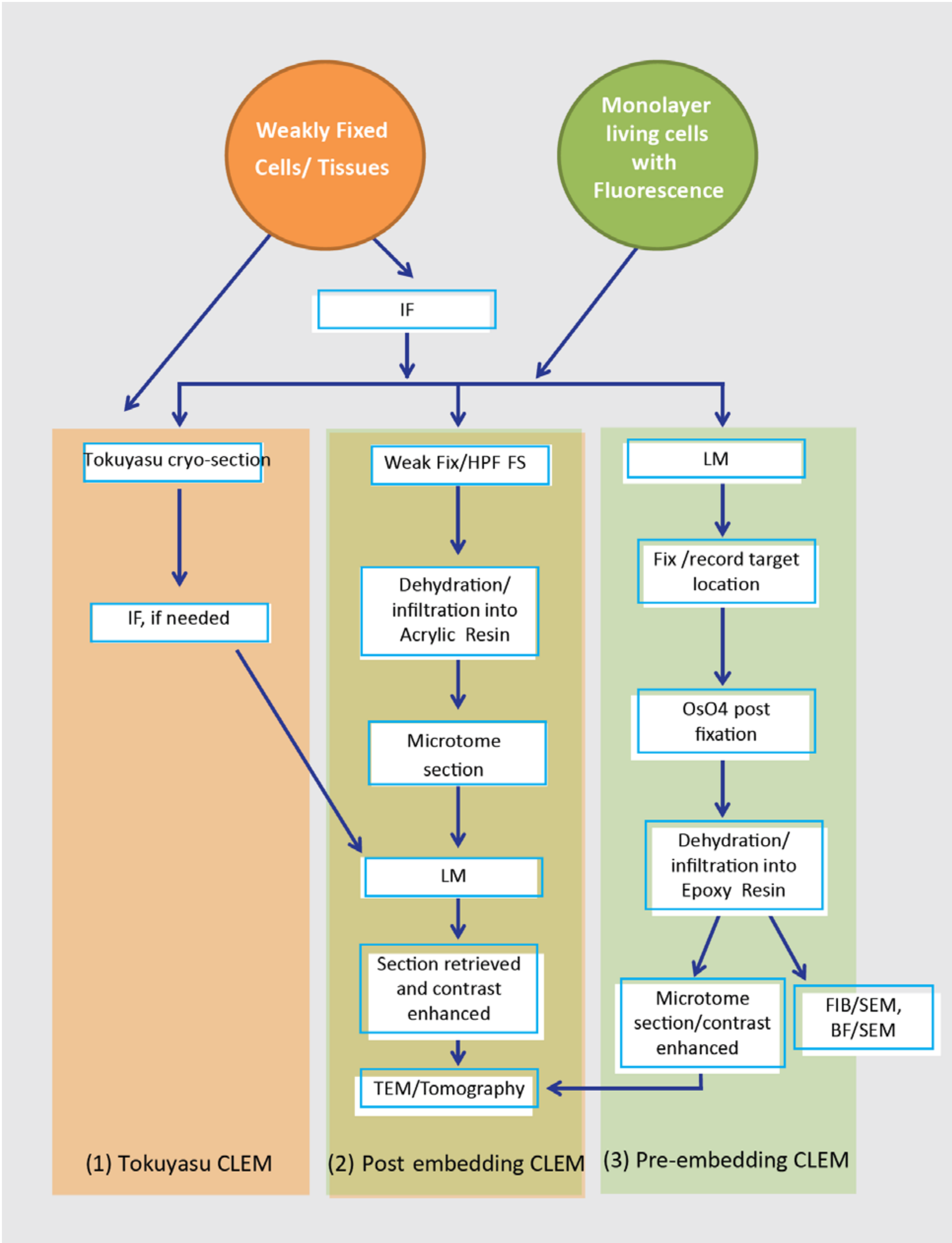

Figure 2 Comparisons of current common markers in CLEM

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 lower brightness • Large size >25 KD for possible perturb the target protein
Synthetic Fluorophore and Particles	• Commercial 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 • Protocol and technical challenges to have good staining and accurate distribution

Figure 3

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 3 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.

References

1. Herderschee, J., et al., *Emerging single-cell technologies in immunology.* J Leukoc Biol, 2015. **98**(1): p. 23-32.
2. Rella, C.E., N. Ruel, and E.A. Eugenin, *Development of imaging techniques to study the pathogenesis of biosafety level 2/3 infectious agents.* Pathog Dis, 2014. **72**(3): p. 167-73.
3. Sridhar, S., et al., *A systematic approach to novel virus discovery in emerging infectious disease outbreaks.* J Mol Diagn, 2015. **17**(3): p. 230-41.
4. Kruger, D.H., P. Schneck, and H.R. Gelderblom, *Helmut Ruska and the visualisation of viruses.* The Lancet, 2000. **355**(9216): p. 1713-1717.
5. Knott, G. and C. Genoud, *Is EM dead?* J Cell Sci, 2013. **126**(Pt 20): p. 4545-52.
6. Renz, M., *Fluorescence microscopy-a historical and technical perspective.* Cytometry A, 2013. **83**(9): p. 767-79.
7. Betzig, E., et al., *Imaging intracellular fluorescent proteins at nanometer resolution.* Science, 2006. **313**(5793): p. 1642-5.
8. Hell, S.W. and J. Wichmann, *Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy.* Opt Lett, 1994. **19**(11): p. 780-2.
9. Gustafsson, M.G., *Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy.* J Microsc, 2000. **198**(Pt 2): p. 82-7.
10. Rust, M.J., M. Bates, and X. Zhuang, *Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM).* Nat Methods, 2006. **3**(10): p. 793-5.
11. Nazerian, K. and H.G. Purchase, *Combined fluorescent-antibody and electron microscopy study of Marek's disease virus-infected cell culture.* J Virol, 1970. **5**(1): p. 79-90.
12. Morgan, C., et al., *A correlative study by electron and light microscopy of the development of type 5 adenovirus. I. Electron microscopy.* J Exp Med, 1960. **112**: p. 373-82.
13. Noda, T., et al., *The importance of the NP: VP35 ratio in Ebola virus nucleocapsid formation.* J Infect Dis, 2011. **204 Suppl 3**: p. S878-83.
14. Martinez, M.G., et al., *Imaging the alphavirus exit pathway.* J Virol, 2014. **88**(12): p. 6922-33.
15. Hellstrom, K., et al., *Correlative light and electron microscopy enables viral replication studies at the ultrastructural level.* Methods, 2015.
16. Larson, D.R., et al., *Visualization of retrovirus budding with correlated light and electron microscopy.* Proc Natl Acad Sci U S A, 2005. **102**(43): p. 15453-8.
17. Spuul, P., et al., *Assembly of alphavirus replication complexes from RNA and protein components in a novel trans-replication system in mammalian cells.* J Virol, 2011. **85**(10): p. 4739-51.
18. Jun, S., et al., *Direct visualization of HIV-1 with correlative live-cell microscopy and cryo-electron tomography.* Structure, 2011. **19**(11): p. 1573-81.
19. Zhang, P., *Correlative cryo-electron tomography and optical microscopy of cells.* Curr Opin Struct Biol, 2013. **23**(5): p. 763-70.
20. Romero-Brey, I., et al., *Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication.* PLoS Pathog, 2012. **8**(12): p. e1003056.
21. Sun, M.G., et al., *Correlated three-dimensional light and electron microscopy reveals transformation of mitochondria during apoptosis.* Nat Cell Biol, 2007. **9**(9): p. 1057-65.
22. Kong, D. and J. Loncarek, *Correlative light and electron microscopy analysis of the centrosome: A step-by-step protocol.* Methods Cell Biol, 2015. **129**: p. 1-18.
23. Verkade, P., *Moving EM: the Rapid Transfer System as a new tool for correlative light and electron microscopy and high throughput for high-pressure freezing.* J Microsc, 2008. **230**(Pt 2): p. 317-28.

24. McDonald, K.L., et al., *Recent advances in high-pressure freezing: equipment- and specimen-loading methods.* Methods Mol Biol, 2007. **369**: p. 143-73.
25. Perkovic, M., et al., *Correlative light- and electron microscopy with chemical tags.* J Struct Biol, 2014. **186**(2): p. 205-13.
26. Peddie, C.J., et al., *Correlative and integrated light and electron microscopy of in-resin GFP fluorescence, used to localise diacylglycerol in mammalian cells.* Ultramicroscopy, 2014. **143**: p. 3-14.
27. Lucas, M.S., et al., *Bridging microscopes: 3D correlative light and scanning electron microscopy of complex biological structures.* Methods Cell Biol, 2012. **111**: p. 325-56.
28. Kukulski, W., et al., *Correlated fluorescence and 3D electron microscopy with high sensitivity and spatial precision.* J Cell Biol, 2011. **192**(1): p. 111-9.
29. Paez-Segala, M.G., et al., *Fixation-resistant photoactivatable fluorescent proteins for CLEM.* Nat Methods, 2015. **12**(3): p. 215-8, 4 p following 218.
30. Watanabe, S., et al., *Protein localization in electron micrographs using fluorescence nanoscopy.* Nat Methods, 2011. **8**(1): p. 80-4.
31. Groos, S., E. Reale, and L. Luciano, *Re-evaluation of epoxy resin sections for light and electron microscopic immunostaining.* J Histochem Cytochem, 2001. **49**(3): p. 397-406.
32. Johnson, E., et al., *Correlative in-resin super-resolution and electron microscopy using standard fluorescent proteins.* Sci Rep, 2015. **5**: p. 9583.
33. Tokuyasu, K.T., *A technique for ultracryotomy of cell suspensions and tissues.* J Cell Biol, 1973. **57**(2): p. 551-65.
34. Slot, J.W. and H.J. Geuze, *Cryosectioning and immunolabeling.* Nat Protoc, 2007. **2**(10): p. 2480-91.
35. Oorschot, V.M., et al., *Immuno correlative light and electron microscopy on Tokuyasu cryosections.* Methods Cell Biol, 2014. **124**: p. 241-58.
36. Takizawa, T. and J.M. Robinson, *Ultrathin cryosections: an important tool for immunofluorescence and correlative microscopy.* J Histochem Cytochem, 2003. **51**(6): p. 707-14.
37. G, J.J., *Cryo-EM, Part A: sample preparation and data collection. Preface.* Methods Enzymol, 2010. **481**: p. xv-xvi.
38. van Rijnsoever, C., V. Oorschot, and J. Klumperman, *Correlative light-electron microscopy (CLEM) combining live-cell imaging and immunolabeling of ultrathin cryosections.* Nat Methods, 2008. **5**(11): p. 973-80.
39. Oorschot, V., et al., *A novel flat-embedding method to prepare ultrathin cryosections from cultured cells in their in situ orientation.* J Histochem Cytochem, 2002. **50**(8): p. 1067-80.
40. Vicidomini, G., et al., *A novel approach for correlative light electron microscopy analysis.* Microsc Res Tech, 2010. **73**(3): p. 215-24.
41. Vicidomini, G., et al., *High data output and automated 3D correlative light-electron microscopy method.* Traffic, 2008. **9**(11): p. 1828-38.
42. Hoboth, P., et al., *Aged insulin granules display reduced microtubule-dependent mobility and are disposed within actin-positive multigranular bodies.* Proc Natl Acad Sci U S A, 2015. **112**(7): p. E667-76.
43. Deerinck, T.J., et al., *Fluorescence photooxidation with eosin: a method for high resolution immunolocalization and in situ hybridization detection for light and electron microscopy.* J Cell Biol, 1994. **126**(4): p. 901-10.
44. Keene, D.R., et al., *Confocal/TEM overlay microscopy: a simple method for correlating confocal and electron microscopy of cells expressing GFP/YFP fusion proteins.* Microsc Microanal, 2008. **14**(4): p. 342-8.
45. Tsien, R.Y., *The green fluorescent protein.* Annu Rev Biochem, 1998. **67**: p. 509-44.
46. Matz, M.V., et al., *Fluorescent proteins from nonbioluminescent Anthozoa species.* Nat Biotechnol, 1999. **17**(10): p. 969-73.

47. Gross, L.A., et al., *The structure of the chromophore within DsRed, a red fluorescent protein from coral.* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 11990-5.
48. Wiedenmann, J., et al., *EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion.* Proc Natl Acad Sci U S A, 2004. **101**(45): p. 15905-10.
49. Shaner, N.C., et al., *Improving the photostability of bright monomeric orange and red fluorescent proteins.* Nat Methods, 2008. **5**(6): p. 545-51.
50. Shaner, N.C., P.A. Steinbach, and R.Y. Tsien, *A guide to choosing fluorescent proteins.* Nat Methods, 2005. **2**(12): p. 905-9.
51. Davidson, M.W. and R.E. Campbell, *Engineered fluorescent proteins: innovations and applications.* Nat Methods, 2009. **6**(10): p. 713-17.
52. Rizzo, M.A., M.W. Davidson, and D.W. Piston, *Fluorescent protein tracking and detection: fluorescent protein structure and color variants.* Cold Spring Harb Protoc, 2009. **2009**(12): p. pdb top63.
53. Giepmans, B.N., et al., *Correlated light and electron microscopic imaging of multiple endogenous proteins using Quantum dots.* Nat Methods, 2005. **2**(10): p. 743-9.
54. Luby-Phelps, K., et al., *Visualization of identified GFP-expressing cells by light and electron microscopy.* J Histochem Cytochem, 2003. **51**(3): p. 271-4.
55. Snapp, E., *Design and use of fluorescent fusion proteins in cell biology.* Curr Protoc Cell Biol, 2005. **Chapter 21**: p. Unit 21 4.
56. Keppler, A., et al., *Labeling of fusion proteins with synthetic fluorophores in live cells.* Proc Natl Acad Sci U S A, 2004. **101**(27): p. 9955-9.
57. Griffin, B.A., S.R. Adams, and R.Y. Tsien, *Specific covalent labeling of recombinant protein molecules inside live cells.* Science, 1998. **281**(5374): p. 269-72.
58. Gaietta, G., et al., *Multicolor and electron microscopic imaging of connexin trafficking.* Science, 2002. **296**(5567): p. 503-7.
59. Adams, S.R., et al., *New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications.* J Am Chem Soc, 2002. **124**(21): p. 6063-76.
60. Karreman, M.A., et al., *Optimizing immuno-labeling for correlative fluorescence and electron microscopy on a single specimen.* J Struct Biol, 2012. **180**(2): p. 382-6.
61. Karreman, M.A., et al., *Discovery of a new RNA-containing nuclear structure in UVC-induced apoptotic cells by integrated laser electron microscopy.* Biol Cell, 2009. **101**(5): p. 287-99.
62. Deerinck, T.J., *The application of fluorescent quantum dots to confocal, multiphoton, and electron microscopic imaging.* Toxicol Pathol, 2008. **36**(1): p. 112-6.
63. Vu, T.Q., et al., *Quantum dots for quantitative imaging: from single molecules to tissue.* Cell Tissue Res, 2015. **360**(1): p. 71-86.
64. Nisman, R., et al., *Application of quantum dots as probes for correlative fluorescence, conventional, and energy-filtered transmission electron microscopy.* J Histochem Cytochem, 2004. **52**(1): p. 13-8.
65. Meisslitzer-Ruppitsch, C., et al., *Photooxidation technology for correlated light and electron microscopy.* J Microsc, 2009. **235**(3): p. 322-35.
66. Sosinsky, G.E., et al., *Markers for correlated light and electron microscopy.* Methods Cell Biol, 2007. **79**: p. 575-91.
67. Maranto, A.R., *Neuronal mapping: a photooxidation reaction makes Lucifer yellow useful for electron microscopy.* Science, 1982. **217**(4563): p. 953-5.
68. Porstmann, B., et al., *Which of the commonly used marker enzymes gives the best results in colorimetric and fluorimetric enzyme immunoassays: horseradish peroxidase, alkaline phosphatase or beta-galactosidase?* J Immunol Methods, 1985. **79**(1): p. 27-37.
69. Li, J., et al., *Membrane targeted horseradish peroxidase as a marker for correlative fluorescence and electron microscopy studies.* Front Neural Circuits, 2010. **4**: p. 6.

70. Pagano, R.E., M.A. Sepanski, and O.C. Martin, *Molecular trapping of a fluorescent ceramide analogue at the Golgi apparatus of fixed cells: interaction with endogenous lipids provides a trans-Golgi marker for both light and electron microscopy.* J Cell Biol, 1989. **109**(5): p. 2067-79.
71. Shu, X., et al., *A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms.* PLoS Biol, 2011. **9**(4): p. e1001041.
72. Martell, J.D., et al., *Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy.* Nat Biotechnol, 2012. **30**(11): p. 1143-8.
73. Grabenbauer, M., et al., *Correlative microscopy and electron tomography of GFP through photooxidation.* Nat Methods, 2005. **2**(11): p. 857-62.
74. Meiblitzer-Ruppitsch, C., et al., *Electron microscopic visualization of fluorescent signals in cellular compartments and organelles by means of DAB-photoconversion.* Histochem Cell Biol, 2008. **130**(2): p. 407-19.
75. Ou, H.D., et al., *Visualizing viral protein structures in cells using genetic probes for correlated light and electron microscopy.* Methods, 2015.
76. Rodriguez, E.A., et al., *New Molecular Tools for Light and Electron Microscopy.* Microsc. Microanal, 2015. **21**(Suppl 3): p. 1-2.
77. Murphy, M.P., *How mitochondria produce reactive oxygen species.* Biochem J, 2009. **417**(1): p. 1-13.
78. Faas, F.G., et al., *Localization of fluorescently labeled structures in frozen-hydrated samples using integrated light electron microscopy.* J Struct Biol, 2013. **181**(3): p. 283-90.
79. Spuul, P., et al., *Phosphatidylinositol 3-kinase-, actin-, and microtubule-dependent transport of Semliki Forest Virus replication complexes from the plasma membrane to modified lysosomes.* J Virol, 2010. **84**(15): p. 7543-57.
80. Veiga, E. and P. Cossart, *Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells.* Nat Cell Biol, 2005. **7**(9): p. 894-900.
81. Rizzo, R., S. Parashuraman, and A. Luini, *Correlative video-light-electron microscopy: development, impact and perspectives.* Histochem Cell Biol, 2014. **142**(2): p. 133-8.
82. Shtengel, G., et al., *Interferometric fluorescent super-resolution microscopy resolves 3D cellular ultrastructure.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3125-30.
83. Heilemann, M., et al., *Super-resolution imaging with small organic fluorophores.* Angew Chem Int Ed Engl, 2009. **48**(37): p. 6903-8.
84. Van Engelenburg, S.B., et al., *Distribution of ESCRT machinery at HIV assembly sites reveals virus scaffolding of ESCRT subunits.* Science, 2014. **343**(6171): p. 653-6.