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Correlative microscopy for structural microbiology

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Understanding how microbes utilize their environment is aided by visualizing them in their natural context at high resolution. Correlative imaging enables efficient targeting and identification of labelled viral and bacterial components by light microscopy combined with high resolution imaging by electron microscopy. Advances in genetic and bioorthogonal labelling, improved workflows for targeting and image correlation, and large-scale data collection are increasing the applicability of correlative imaging methods. Furthermore, developments in mass spectroscopy and soft X-ray imaging are expanding the correlative imaging modalities available. Investigating the structure and organization of microbes within their host by combined imaging methods provides important insights into mechanisms of infection and disease which cannot be obtained by other techniques.

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Introduction

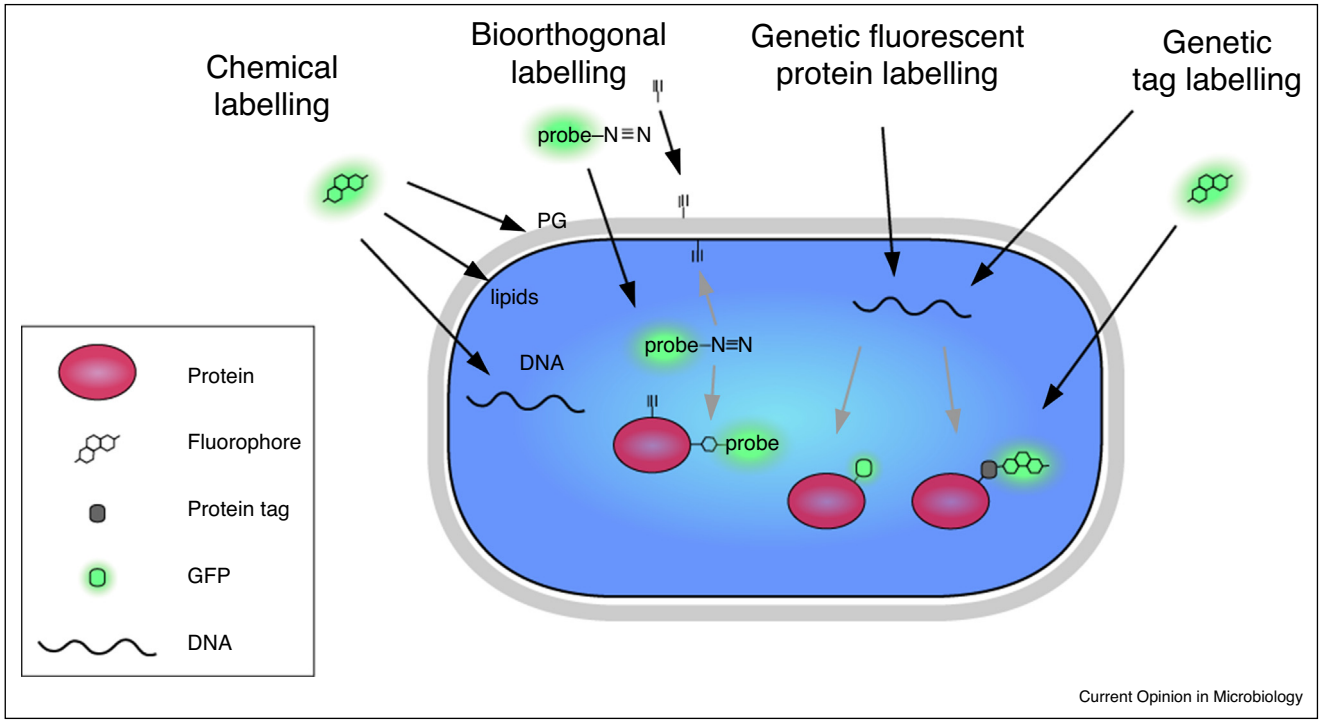
All infections have their origins in the invasion and proliferation of microbes in their host. Visualization of bacteria and viruses in their native state and within their natural environment generates valuable structural, functional and organizational information about infection and disease. Observation of infectious agents inside their hosts brings about several challenges due to the difference in length scales between the environmental context, namely host cells and tissues, and the relatively small bacteria and viruses. Correlation of multiple imaging technologies allows for this scale discrepancy to be overcome. Often light microscopy (LM) is used to identify and localize the objects of interest inside large volumes, and electron microscopy (EM) is used to image their structural details. Although combinations of different LM and EM techniques can be used to image microorganisms, common steps in any correlative LM and EM (CLEM) workflow include labelling and identification of the microbes, localization and navigation to these microbes within the electron microscope, and high-resolution imaging. CLEM imaging provides valuable information about the interactions between host and pathogen *in situ* at a macromolecular level, which is often not obtainable by other techniques.

Identifying organisms and molecules of interest by specific labelling

Fluorescent labelling of structures is important for CLEM imaging and can be achieved in several ways (Figure 1). Non-genetic labels, where chemically conjugating fluorescent dyes to structures such as lipids, proteoglycans, or nucleotides, or to illuminate specific cell activities such as mitochondrial membrane potential [1], can target whole microbes, certain structures, or functional sites inside microbes. Celler *et al.* have used lipid and peptidoglycan dyes and fluorescent light microscopy (fLM) to demonstrate the formation of novel cellular compartmentalization assemblies as well as sites of cell wall formation within *Streptomyces coelicolor* [2]. This chemical labelling is straightforward and generates robust signal, but its specificity and applicability is limited.

Genetically encoding a fluorescent label that is fused to the protein of interest is the most commonly used technique for targeting specific proteins, due to the ubiquity of optimized labels and the availability of extensive protocols [3]. Genetic labels now are easier to introduce with current genome editing technologies [4], providing identity information that complements the ultrastructural information provided by EM. Positive identification and targeting of a particular microbe *in situ* by fLM has been used with great success [5,6••,7•] and the reader is referred to excellent existing reviews and protocols for further information [8,9,10••,11,12••,13]. Briefly, the general approach is to insert the gene for a fluorescent protein, often green fluorescent protein (GFP) or a variant, fused to a protein of interest through a short, flexible linker. Fluorescent imaging of the GFP-tagged protein, expressed by the microbe, is then used for identification and targeting of suitable regions for morphological EM imaging inside the host. Strauss *et al.* precisely localized HIV-1 assembly and budding from infected cells by live-cell imaging, and used cryo-electron tomography (cryo-ET) to resolve the structure of individual virus particles and their membrane tethers [14]. One should be aware that fluorescent protein tagging might interfere with

Figure 1



Fluorescent labelling strategies. Fluorescent labelling strategies for microorganism constituents include chemical labelling for DNA, lipids and PG, bioorthogonal labelling for PG, lipids and proteins, genetic fluorescent protein fusion and genetic tag labelling for proteins.

expression, localization or functionality of the labelled protein [15] and possible incompatibilities with staining methods used for EM [16].

Additionally, genetically encoding (small) chemical labels onto proteins, which are later coupled to synthetic fluorophores, has the advantage that these interfere less with protein functionality. They also tolerate better the embedding and staining protocols for high pressure freezing and freeze substitution to circumvent fluorescent quenching often observed for GFP [17,18].

Genetic manipulation to introduce the label is limited to proteins. To expand beyond protein labelling, alternative bioorthogonal approaches have been developed. The advantage of bioorthogonal labelling is that non-protein biomolecules such as nucleic acids, glycans and lipids can be labelled [19]. Bioorthogonal labelling utilizes the insertion of small chemical moieties, which are inert (and thereby invisible) to normal biochemical reactions, and their subsequent selective reaction to incorporate a specific tag [20,21••]. Bioorthogonal fluorescent tags have been used to identify *E. coli*, intact or partially degraded, in regions using general morphology that is sufficiently distinct from the host [22]. Genetic code expansion to incorporate unnatural amino acids allows proteins that contain the unnatural amino acid (in practice all proteins from modified microbes are assumed to be labelled) from a single organism to be identified within a mixture of species [23,24]. The drawback is the extensive genetic manipulation that is required to expand the genetic code used by the cell. Introduction of labels that are capable of diaminobenzidine (DAB) polymerization, which can be directly detected in the EM, can be used when targeting within the EM micrograph needs to be more accurate than the LM-EM correlation accuracy (see section Integrating information to overlay and annotate volumes) [21••].

Another approach to identify organisms and their subcellular content in correlation with EM imaging is chemical isotope detection, either by nano-secondary ion mass spectrometry (NanoSIMS) [25,26,27•] or Energy-dispersive X-ray spectroscopy (EDX) [28]. These techniques are used for correlative identification but not targeting of structures. Specific isotopes or elements can be imaged by NanoSIMS with sensitivities in the parts-per-million range [27•] and a lateral resolution of around 50 nm [29]. All elements and isotypes can be detected, and it is especially useful in cases where an isotope can be constrained to a particular species by pre-culturing in stable isotopes. It allows for following the movement of minerals within a microbial community [30], intracellular drug trafficking [31] or can be used in a pulse-chase

experiment to monitor turnover [25]. Additionally, multiplexing is relatively straightforward as multiple elements and isotopes can be reliably detected with minimal complications to sample preparation, thus allowing multiple microbes to be identified within the same volume.

Selection of an appropriate labelling technique to identify a particular microbe will depend ultimately on the biological system and question at hand. From the numerous techniques, fLM will likely be the most common choice in the future. NanoSIMS and bioorthogonal techniques may be important in the future and offer many important opportunities for further research. Exceptions for non-protein biomolecules and cases where genetic fusions are not possible may require another approach such as immunolabeling [32] or isotope distinctions.

Imaging large volumes efficiently

LM guided and targeted imaging of specific structures is paramount for efficient imaging, since current EM imaging is too slow to make imaging the whole volume feasible. Additionally, the volumes that are routinely imaged with LM cannot be directly imaged with EM. Electrons can travel a relatively small distance through a sample unscattered (±0.1 μm) compared to light (±1 mm). Thus, the physical limit of sample thickness for imaging is much thinner for EM than for LM [33]. To overcome this limit, volumes are sampled using sections or by blockface imaging, where thin layers are sequentially removed from a tissue block and the newly exposed surfaces are imaged. This sampling allows the cell morphology and the structures surrounding the targeted microbe to be imaged within a volume that is larger than the physical limit of EM imaging.

For EM imaging, traditionally, thin serial sections are cut from a block of resin-embedded sample and are placed onto a grid with a supporting carbon layer for TEM. Automated sectioning systems allow for the collection and tracking of hundreds of sections (±75 nm) on silicon wafers [34•], or indium tin oxide (ITO) coated glass, which are imaged by SEM. This technique is called array tomography and volumes are obtained by computationally joining the 2D images with a spacing corresponding to the section thickness. The use of automated systems and intelligently combining low-resolution scans with targeted high resolution imaging only where necessary speeds up imaging millimetre sized samples [34•,35]. The advantage of serial sectioning for CLEM imaging is that the sections can be used to target specific regions in the block if the sample preparation preserves the fluorescent signal within the final sections, as was done for vaccinia infected cells [6••], or by on section labelling [22].

SEM images the surface of a tissue block, which can be removed by slicing with a diamond knife or ion-beam [36]. By iterating the imaging and the removal of the surface, large volumes can be sampled. The time needed to prepare and image the many surfaces forces compromises between the total volume that is imaged and the final resolution [37••]. The new surface can be exposed by cutting with a diamond knife, as was done to investigate the infection of zebrafish with *Mycobacterium marinum* [38], or by using a focussed ion beam (FIB) to ablate the surface [39]. Diamond knives are generally faster to expose the new surface, while removing more material with each slice, around 50 nm. This allows larger volumes to be imaged, but with lower axial resolution. The minimum thickness for FIB milling is usually dependent on the depth of the beam damage caused while imaging. Time gains can be made by using multi beam approaches [40], particularly for samples that have large surface areas compared to their depths, but it is less suitable for volumes that require many slices where exposing the new surfaces takes as long as imaging. Blockface methods also do not allow for revisiting of regions of interest as the sections are lost after each removal.

Methods to image thicker samples, thereby reducing the need for sectioning, utilize soft X-rays (high energy photons) with higher penetration depths for imaging than electrons. Soft X-rays were used to image whole eukaryotic cells while resolving their ultrastructural features at 50 nm resolution in a correlative soft X-ray/fLM setup [41]. Compared to serial EM imaging, sample handling is simplified in this technique and it requires much less data acquisition time. However, the infrastructure and instrumentation requirements are significant.

Micro-CT also allows for thick samples (a few millimetres) to be imaged with hard X-rays. In cases where X-ray dose is not a consideration, such as with metal stained samples that are similarly processed as for EM, it is possible to reach sub-micrometre pixel sizes in these much larger volumes. This allows for more constrained targeting of the high-resolution EM imaging to a smaller volume [42]. However, the current resolution limitations prevent imaging features smaller than 1 μm, limiting its present applicability for microbes.

The techniques for imaging large volumes by EM allows for the visualization of complete cellular and tissue morphology. However, CLEM targeted imaging around sites of microbe infection are highly desired for efficiency. Future improvements will mostly come from better automation and integration of LM and EM techniques along with improved specimen preparation, rather than fundamental changes to the integrating workflows or changes in the physical principles utilized. These improvements will increase the amount of data collected dramatically, raising the additional challenges of integrating and analysing the large amounts of data.

Integrating information to overlay and annotate volumes

Combining the datasets generated by multiple imaging modalities for CLEM purposes is crucial. While it is conceptually simple to overlay two different datasets, accuracy and the time investment needed are important issues when working with different resolution datasets. The resolution of conventional fLM is a few orders of magnitude lower than EM, limiting its correlation accuracy to the EM data, which it generally around 50–100 nm using bead based approaches [12••,43,44]. The use of photoswitchable proteins that allow for super-resolution LM [6••,45,46] in combination with fiducial markers to precisely overlay the images [12••] can improve the correlation to 10–20 nm [46,47]. In ideal cases, integrated microscopes can improve the accuracy to better than 5 nm [48]. Thus, depending on the size of the microbe of interest and of the targeted region, as well as how crowded the environment is and the precision needed, different fluorescent tags should be considered for correlative studies to ensure that the overlay is sufficiently accurate.

Combining different LM and EM datasets has dual purposes. The first is where fLM is used to guide EM data collection. In some cases the specimen shape itself can be used for correlation, providing the shape is obvious and the target can be observed in both LM and EM [2]. In many cases, it is necessary to locate fiducials (or other landmarks) that are visible in both modalities, such as fluorescent beads. These can serve as landmarks to calculate the required coordinate transformations and locate the exact region of interest in the electron microscope from the LM image with measurable accuracy [44]. Alternatively, the commonly used EM stain uranyl acetate can be made fluorescent under cryo conditions and can be directly correlated with the EM image [49]. Having the LM images available within the EM reference frame is particularly useful when targeting rare events or when setting up long EM acquisition runs to ensure the appropriate regions are imaged. Specialized EM grid geometry (e.g. finder grids) and patterned substrates [7•] allow for easy coordinate transformations [7•,50]. Targeting is also critical for block face or other destructive methods where it would not be possible to revisit a region. Commercial systems have been developed that use markers on the sample carrier or user-identified points to perform the geometrical transformations [12••,51].

The second need for combining LM and EM is for post-acquisition visualization. For simple overlay of two-dimensional images, nonspecialized software may be sufficient. However, to combine datasets of different types, modalities dimensions and sizes, specific imaging packages such as Amira (Thermo Fisher Scientific, Waltham, MA), Icy [52] with the eC-CLEM plugin [53•], OMERO [54], ImageJ [55] with plugin TrakEM2 [56], and others bundled with the Fiji distribution [57,58] are necessary. Especially the handling of very large individual datasets (e.g. a sample of 50 μm^3 recorded using 1 nm^3 voxel sizes with 8-bit voxel values would require ~113 terabytes of storage) underscores the need for smart algorithms and significant computing resources for CLEM imaging.

Analysis and interpretation of the combined datasets requires that features of interest are accurately identified, and is a critical part of fully utilizing the information. Segmentation and annotation of features remains a great challenge and using generalized algorithms is difficult, primarily because structural features depend on particular sample preparation and imaging conditions. This remains a labour-intensive, manual process. Automated methods are under development, and some advanced automatic segmentation methods are available for tracing and identifying cells in neuronal tissue [59,60]. Automatically identifying cells in other tissues, or sub-cellular structures is lagging. There has been some exciting progress in neural networks [61•] that can be trained to identify particular features of interest, and should be easily applied to well defined structures, for example some viruses. The large structural variation between and within organelles and other objects (i.e. cytoskeletal elements, ribosomes, gap junctions, and so on) that could surround any given microbe, make this an extremely challenging problem.

Conclusion

Effective correlative imaging, that is, using imaging modalities that span large size and resolution ranges, enables identification, localization and visualization of microbes within the ultrastructural context of their natural host environment. Localizing viruses or bacteria within their larger environment requires specific labelling, sample preservation that serves both imaging modalities, and thinning (or repeated surface removal) for EM. Automated serial sections and data acquisition now allow for larger volumes to be imaged and more easily integrated with LM data, and is expected to provide a wealth of information to the microbiology field. Improvements to LM that may be applied to microbial research in the immediate future include the use of two objective lenses to increase the amount of light collected (and thereby improve the resolution) [62], and the increased use of single molecule localization techniques. Long term, techniques such as microCT and soft X-rays may become feasible to look at larger volumes at high resolution, but currently only EM can provide information in the 1 nm resolution regime.

For the future, CLEM imaging of microbes should be improved by taking LM tagging of microbes a step further. Specifically, new strategies for tagging microbes while they are in their native environment need to be

developed. In current studies, infected cell cultures and *ex vivo* microbe–host systems are generally used. Tagging of microbes that are already inside their host, that is, *in vivo* or *in situ*, for identification and targeted imaging, is a great challenge, but it is extremely important to get a realistic and unperturbed view of naturally occurring infections. Furthermore, large scale correlative EM imaging that pushes towards the goal of millimetre scales should be taken to the next level in terms of speed, integration of modalities, reconstruction and data mining. Currently LM imaging, SEM/TEM imaging, volume reconstruction, data mining and visualization are separate processes which are sequentially knitted together. The particular rate limiting step varies by application, but it is often in the reconstruction, data mining or visualization steps, rather than in the actual imaging, a trend that is likely to increase as imaging becomes increasingly automated. While less imaging time is always desirable, in the future efficiently making sense of all the data will require more effort than actually obtaining the raw data. All of these steps should be approached holistically, and integrated into a single working system, in order to drastically increase overall speed and usability. Taken together these developments should enable efficient, functional and structural identification of infections *in vivo*.

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