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Current Opinion in Microbiology

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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Current Opinion in Microbiology 2018, **43**:132–138

This review comes from a themed issue on **The new microscopy**

Edited by **Stephan Uphoff** and **Ariane Briegel**

https://doi.org/10.1016/j.mib.2018.01.009

Introduction

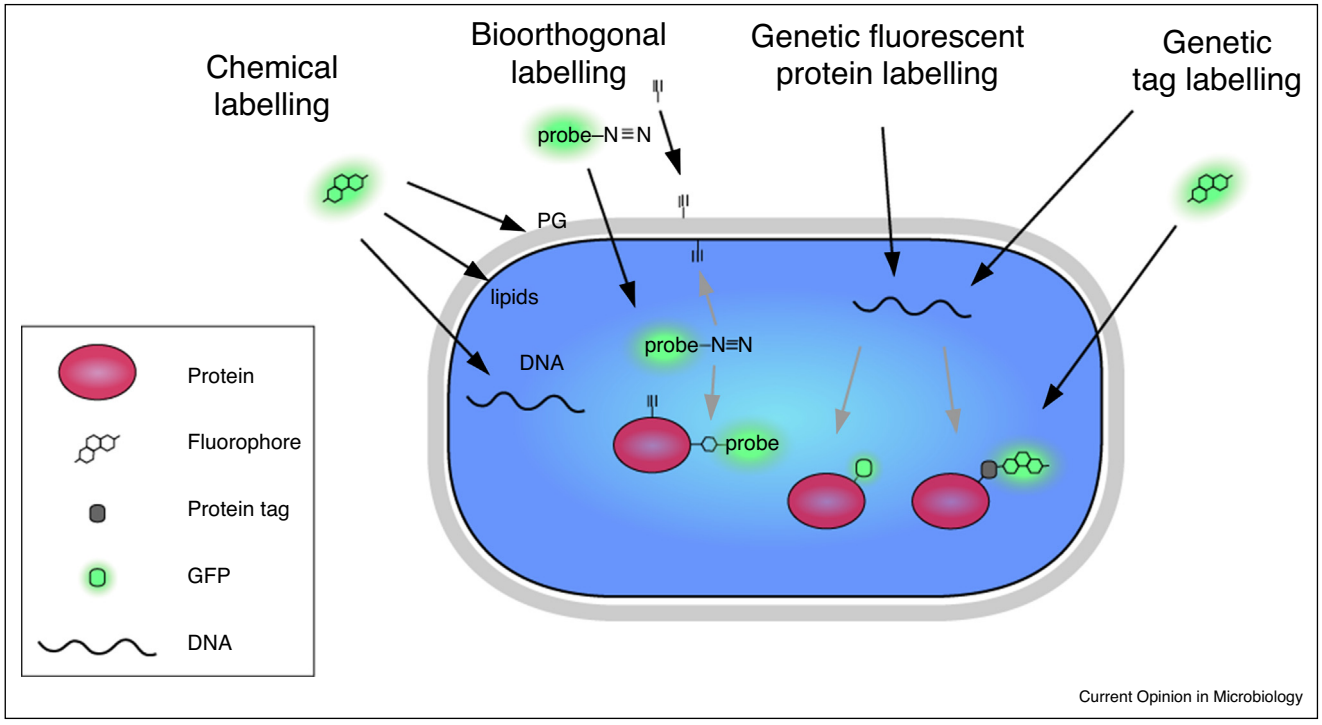
All infections have their origins in the invasion and proliferation of microbes in their host. Visualization of bacteria and viruses in their native state and within their natural environment generates valuable structural, functional and organizational information about infection and disease. Observation 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