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Review

The Pathogen–Host Interface in Three Dimensions: Correlative FIB/SEM Applications

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Pathogens survive and propagate within host cells through a wide array of complex interactions. Tracking the molecular and cellular events by multidimensional fluorescence microscopy has been a widespread tool for research on intracellular pathogens. Through major advancements in 3D electron microscopy, intracellular pathogens can also be visualized in their cellular environment to an unprecedented level of detail within large volumes. Recently, multidimensional fluorescence microscopy has been correlated with volume electron microscopy, combining molecular and functional information with the overall ultrastructure of infection events. In this review, we provide a short introduction to correlative focused ion beam/scanning electron microscopy (c-FIB/SEM) tomography and illustrate its utility for intracellular pathogen research through a series of studies on *Shigella*, *Salmonella*, and *Brucella* cellular invasion. We conclude by discussing current limitations of and prospects for this approach.

Highlights

A current challenge in studying pathogen–host interactions is understanding how molecular players and cellular mechanisms function in the three-dimensional cellular environment at subdiffraction resolution.

FIB/SEM is an emerging technique that can be used to investigate expansive three-dimensional cellular interfaces between pathogen and host at ultrastructural resolution.

The correlation of FIB/SEM with fluorescence microscopy (c-FIB/SEM) enables the study of transient or rare infection events while also providing molecular specificity within the ultrastructural landscape.

Recently, c-FIB/SEM has revealed fundamental aspects of the mechanisms of cellular invasion by *Shigella*, *Salmonella*, and *Brucella*.

c-FIB/SEM is becoming an indispensable tool for pathogen research as it can provide a wealth of biological information not obtainable by other technologies.

Studying Pathogens with Light and Electron Microscopy

Infection by pathogens occurs at a complex and dynamic interface between the pathogen and host that has been shaped over millions of years of evolution in a so called 'arms race' [1]. The mechanisms of infection and how they can be exploited against the invading pathogen are subject to intensive biological and medical research. Infection by invasive bacteria consists of host cell invasion, residence within the host cell, pathogen replication, propagation to neighboring cells and evasion of the host immune response, all requiring a finely tuned pathogenic strategy that manifests at the pathogen–host interface [2,3] Many of the molecular players and general mechanisms employed at this interface have been identified and characterized in some detail. However, how these function in the three-dimensional cellular environment at subdiffraction resolution often remains poorly understood.

Microscopy has been instrumental in the study of intracellular pathogens for more than 50 years. Light microscopy approaches were, and remain, extremely powerful for this purpose. Fluorescence microscopy provides localization information on specific fluorescently labeled molecules involved in infection processes [often with autofluorescent proteins like the green fluorescent protein (GFP)], and its combination with multidimensional imaging enables dynamic insights to where and when the host and pathogen factors cross-talk [4]. It also provides functional information through the development of a multitude of cellular reporters that are able to capture an ever-growing number of physical parameters [5]. Fluorescence microscopy, however, is limited in its ability to fully describe the three-dimensional cellular architecture of the pathogen–host interface due to both its resolution limit and the visualization of just a few fluorescent tags (normally two or three) against a 'black' background lacking cellular context.

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Classic electron microscopy (EM), with its superior resolving power and 'ultrastructural view' of cellular environments (containing information about membrane, organelle, and compartment organization based on heavy-metal staining), has historically been a major driving force for the study of pathogens, either as individuals or within infected host cells. Electron cryotomography (cryoET), a technique capable of imaging pristinely preserved samples at very high resolution, is often applied to the study of isolated (i.e., outside the host cell) intracellular pathogens, particularly viruses [6]. This technique has recently emerged as an exciting tool for determining the atomic structure of proteins and complex macromolecular machines in their native vitrified state, and is increasingly used for studying cellular environments using highly specialized pipelines requiring a substantial time investment [7–9].

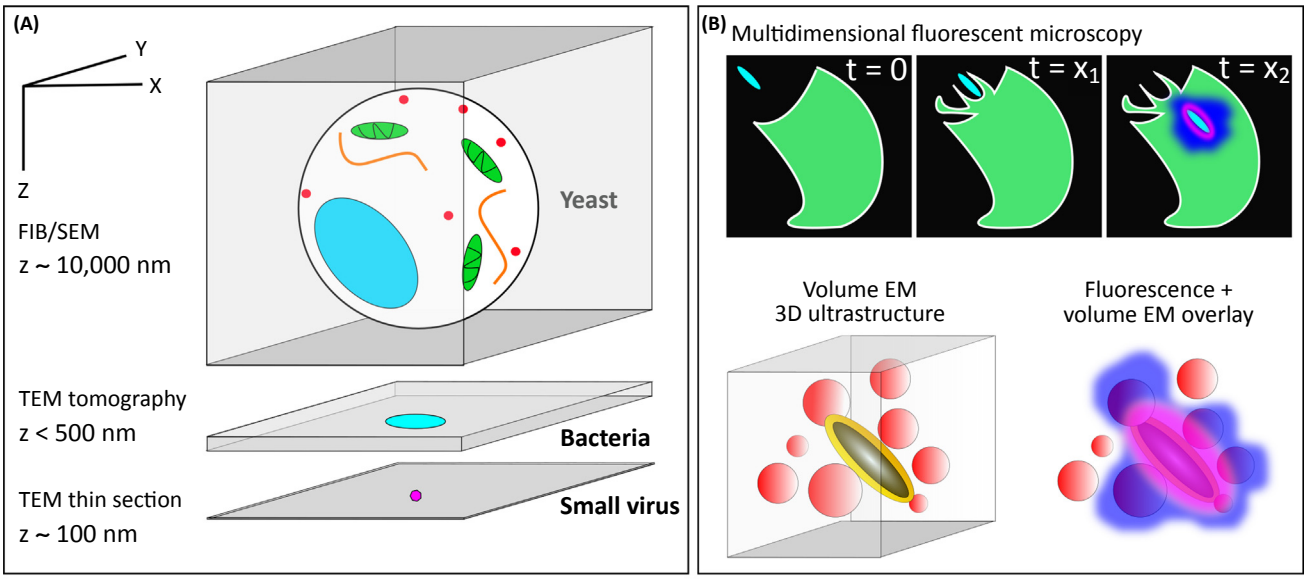
Until recently, most EM techniques were constrained in their ability to fully describe expansive three-dimensional cellular interfaces between pathogen and host, due to limits of acquisition volume, normally confining sample thickness to under 500 nm. In practice, this meant that studying the ultrastructural organization of cells or pathogen–host interfaces with a thickness larger than approximately the width of a single bacterium was highly challenging and required serial sections or serial tomography studies that are laborious and require tremendous skill [10]. In the past decade or so, new 'volume EM' techniques that are not constrained by these limits have been developed. These include serial block face scanning electron microscopy (SBF-SEM), array tomography, and focused ion beam/scanning electron microscopy (FIB/SEM) tomography, all of which allow the investigation of much larger cellular environments containing extensive pathogen–host interfaces with relative ease [11] (Figure 1A).

A major challenge when using EM, including volume techniques, is the identification of transient or rare events that take place during infection, as these are often not synchronized between cells and can take place in the order of several minutes or less. This issue can be addressed by the application of fluorescence microscopy and volume EM to the same specimen via correlative light-electron microscopy (CLEM), giving access to discrete events during infection [12,13]. Acquired fluorescence microscopy and volume EM datasets can ultimately be precisely overlaid computationally, yielding molecular specificity through fluorescence within the global three-dimensional ultrastructural cellular context, another important advantage of this approach. (Figure 1B). The application of volume EM in CLEM pipelines has the potential to play a key role in the development of novel concepts at the pathogen–host interface. Below, we present a series of studies on bacterial pathogens, using this approach, that have already led to important findings relating to the intracellular life styles of *Shigella, Salmonella*, and *Brucella*.

Technical Overview: Correlative FIB/SEM

FIB/SEM tomography (most often refered to only as FIB/SEM) is a volume EM technique in which large-volume tomograms are acquired using a focused ion beam/scanning electron microscope [14]. Briefly, the principle of FIB/SEM is that an embedded biological sample is exposed to a focused ion beam capable of removing a thin layer of material (5–10 nm thick) in a highly precise manner termed 'milling'. Between each sample milling, a scanning electron beam is used to image the newly exposed surface. By repeating this process hundreds or even thousands of times, a large sample volume (of the order of 1000 μm^3) can be acquired with up to 5 nm resolution in all axes. The high axial (z) resolution of FIB/SEM is a particular advantage of this technique compared to other volume EM techniques [11].

Datasets produced by FIB/SEM are usually around 2 gigabytes in size and can be thoroughly examined from any orientation and subjected to detailed quantitative analysis using software such as Amira or Avizo (Thermo Fisher Scientific) running on a strong computer equipped with a



(C)

Stage	Biological experiment	Light microscopy	Sample preparation for FIB/SEM	FIB/SEM acquisition	Data alignment and filtering	3D correlation & visualization
Time	Variable	~ 1 day	~ 1 week	1-2 days	~ 1 day	Variable
Method		Gridded cover slips or sapphire disc	Standard chemical or HPF/FS preparation		Standard image processing software	3D visualization software

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Figure 1. Volume EM and Correlative Light-Electron Microscopy (CLEM). (A) Acquisition volume of volume EM compared with other electron microscopy (EM) techniques. Thin-section transmission electron microscopy (TEM) is normally performed on samples with a thickness of 70–100 nm, and conventional tilt-series EM tomography is normally limited to samples no thicker than 500 nm, whereas a sample thickness of 10 microns (i.e., 10 000 nm) is standard for focused ion beam/scanning electron microscopy (FIB/SEM) acquisition with high resolution in all axes. Larger acquisition volumes are possible using other volume EM techniques, albeit with unequal resolution in all axes. For each EM technique compared, the standard sample thickness (z) is presented together with an example of an organism that fits into each volume. (B) Advantages of correlative volume EM for the study of pathogens. Transient or rare events such as the entry step of a bacterial pathogen can be identified using multidimensional fluorescence microscopy. Specific labels provide information about the location of the bacteria (cyan), actin recruitment (magenta) and host molecules recruitment to the entry site (blue) (top). The same entry site is then imaged using volume EM providing 3D quantitative ultrastructural information, for example the presence of a bacterial containing vacuole (yellow) and surrounding vesicles (red) (bottom left). Finally, the fluorescent signals are combined with the volume EM data to provide molecular information within the ultrastructural volume, for example revealing that some of the vesicles surrounding the bacteria are labeled with host molecules (blue/red overlay) while some are not (bottom right). (C) Example of a typical c-FIB/SEM experimental pipeline with approximate timelines and methods used. Samples can be prepared by standard chemical fixation and dehydration or by high-pressure freezing and freeze substitution (HPF/FS).

modern graphics card. Such analysis can provide information about the number, shape, 3D spatial distribution, connectivity, and integrity of cellular structures surrounding (and within) pathogens. The ability of FIB/SEM to quantitatively address questions related to membrane connectivity and integrity is of particular importance for studying the pathogen–host interface. Many intracellular pathogens subvert host endocytic compartments to enter the host cell, alter the trafficking and integrity of these compartments at specific stages of infection, and communicate with other host cellular organelles such as the Golgi apparatus or the endoplasmic reticulum (ER) [15–18].

FIB/SEM has been successfully combined with three-dimensional fluorescence microscopy using CLEM approaches [19–26]. Correlative FIB/SEM (c-FIB/SEM) allows transient or rare

biological events to be pinpointed by fluorescent labels prior to FIB/SEM acquisition, and these fluorescent signals can later be correlated to fine three-dimensional details within the ultrastructural volume (often discernable due to the high axial resolution), providing molecular labeling to the observed structures. Therefore, the defining feature of c-FIB/SEM is its ability to provide access to intricate structural detail placed within a much broader cellular context at chosen sites and time-points.

Many human pathogens have already been subjected to investigation using either stand-alone FIB/SEM or c-FIB/SEM, including viruses, bacteria, parasites, and fungi (Table 1).

Experimental Workflow for c-FIB/SEM

In the first step of a typical c-FIB/SEM pipeline (Figure 1C), cells targeted for infection are grown on a gridded glass-bottomed petri dish that allows the precise identification of each invasion event within a coordinate system. After infection, samples are fixed, and invasion sites are imaged by 3D multichannel fluorescent microscopy using a high numerical aperture objective. Using confocal microscopy or a super-resolution imaging approach at this step can be advantageous, as these methods can capture important biological details that may be obscured with other techniques.

Table 1. Overview of FIB/SEM Studies of Human Pathogens

Category	Pathogen	Technique	Refs
Viruses	HIV type I	FIB/SEM	Bennet *et al*. [68]
			Felts *et al.* [69]
			Do *et al*. [70]
	HIV type I	c-FIB/SEM	Wang *et al*. [71]
			Murphy *et al.* [20]
	Human cytomegalovirus	FIB/SEM	Villinger *et al.* [72]
Bacteria	*Staphylococcus aureus*	FIB/SEM	Gu *et al.* [73]
	Mixed population	FIB/SEM	Oberbach *et al.* [74]
	Shigella flexneri	c-FIB/SEM	Mellouk *et al*. [22]
			Weiner *et al.* [25]
	Salmonella enterica	c-FIB/SEM	Santos *et al.* [46]
			Fredlund *et al.* [45]
	Mycobacterium avium	c-FIB/SEM	Beckwith *et al.* [23]
	Mycobacterium tuberculosis	FIB/SEM	Ellis *et al.* [75]
	Brucella melitensis *Brucella abortus*	c-FIB/SEM with structured illumination	Sedzicki *et al.* [51]
Parasites and fungi	*Plasmodium falciparum*	FIB/SEM	Weiner *et al.* [29]
			Kapishnikov *et al.* [76]
			Medeiros *et al*. [77]
	Trypanosoma cruzi	FIB/SEM	Alcantara *et al*. [78]
			Vidal *et al*. [79]
	Toxoplasma gondii	FIB/SEM	Martins-Duarte *et al.* [80]
	Cryptococcus neoformans	FIB/SEM	Ramos *et al.* [81]
	Candida albicans	FIB/SEM	Weiner *et al.* [82]

Samples are then further processed for FIB/SEM acquisition. They can be prepared using routine EM protocols that include chemical fixation, staining with heavy-metal salts, dehydration and embedding in plastic resin, or alternatively high-pressure freezing followed by freeze-substitution and resin-embedding, requiring the use of sapphire discs instead of glass-bottomed petri dishes [26–30]. In the studies discussed below, samples were prepared by using standard EM chemical fixation methods, facilitating simple workflows and relatively fast turnaround times, albeit with reduced sample preservation compared to 'cryo-fixation' approaches. Finally, the resin-embedded samples are coated with a thin metal layer and placed within the focused ion beam/scanning electron microscope, where the coordinate system, now imprinted on the resin surface, allows quick identification of sites of interest. Once the precise location and orientation of the targeted cell (already imaged by three-dimensional fluorescence microscopy) have been defined, and depending on acquisition parameters and the sample volume required, datasets are produced within a few hours to several days, using a fully automated acquisition process. After alignment and contrast adjustment of the FIB/SEM data, it can be precisely overlaid with the three-dimensional fluorescence microscopy data of the same site using specialized software, such as Amira. Fiducials for the merging of the datasets can be either the invading pathogen, host compartments, or added ones like quantum dots. Finally, the same software is used to segment (represent in three dimensions) the structural details inside the FIB/SEM volume and the links between the two datasets. Segmentation is often the most time-consuming step in the c-FIB/SEM pipeline, and while currently normally performed either manually or using semiautomated tools, new computational approaches promise to drastically improve the time and effort involved in this step (see below under the heading 'c-FIB/SEM Future Perspectives and Challenges').

Application of c-FIB/SEM to the Study of Invasive Bacteria

Invasive bacteria, such as *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, *Salmonella enterica*, and *Shigella flexneri*, are capable of inducing their own uptake into host cells within bacterial containing vacuoles (BCVs). Then, some of them dwell within this endomembrane-bound compartment (e.g., *Salmonella*), whereas others (e.g., *Shigella* or *Listeria*) damage the BCV to access the host cytoplasm. They employ an array of bacterial effectors that act to subvert host pathways and compartments, allowing their own propagation and evasion of the host immune system. The life cycles of many invasive bacteria have been described using classic electron microscopy [31–33]; however, many fundamental questions about the progression of invasion and interaction with the host remain. Especially, understanding the formation of the intracellular niche of the different pathogens requires an in-depth understanding of which host compartments are involved – how do the pathogens engage, for example, with the endocytic compartment, the Golgi apparatus, or the ER? These cellular structures can be readily identified by specific fluorescence markers, and FIB/SEM provides in-depth information on their alterations or potential damage (such as vacuolar rupture) during the process of infection.

Shigella: Towards a Vacuolar Rupture Mechanism

Shigella flexneri is a Gram-negative bacterial pathogen that is the causative agent of bacillary dysentery. *S. flexneri* is transmitted through fecal–oral contamination, and upon ingestion it can invade colonic mucosa, eliciting an intense inflammatory reaction and tissue destruction [34]. Invasion begins when *S. flexneri*, in contact with the gastrointestinal tract epithelium, injects bacterial effectors – via a type III secretion system – that induce host membrane ruffling and uptake into the host cell. Quickly, after internalization (within ~10 min), the pathogen escapes its phagocytic vacuole [35] and directionally polymerizes host actin, allowing it to propagate into neighboring cells [36]. The general paradigm of *S. flexneri* invasion is centered on a macropinocytotic-like uptake mechanism, whereby injected bacterial effectors hijack the cell's actin

polymerization machinery and induce membrane ruffling that then acts to capture bacteria, allowing their uptake and internalization [37]. Many open questions regarding the mechanism of bacterial internalization and, in particular, the vital step of vacuolar rupture and escape, remain. We have employed an array of cell biology tools in combination with c-FIB/SEM to examine the early steps of *Shigella* invasion in detail [22,25]. *S. flexneri* entry, vacuolization, and vacuolar rupture are highly transient events (of the order of minutes), making access to each discrete step for ultrastructural investigation a daunting task unless events are first detected by light microscopy. Specific fluorescent probes for the successive cellular events during *Shigella* entry provided precise access to each invasion step. Consequently, this was exploited in a CLEM pipeline using FIB/SEM acquisitions at the exact same site. This approach provided not only access to the transient, rare events, but gave also molecular specificity to the FIB/SEM data upon combination of the light and electron microscopy datasets (Figure 2).

c-FIB/SEM of very early *S. flexneri* invasion [25] (prior to vacuolar rupture) revealed two compartments at the invasion site – a tight, uniform BCV, closely enveloping the bacteria inside, and multiple vesicles presenting a large variation in size surrounding the BCV. This observation stands in stark contrast to the prevailing entry model in which *S. flexneri* enter host cells in heterogeneous macropinocytic pockets formed due to membrane ruffling [38]. By imaging *S. flexneri* invasion in the presence of fluorescent dextran acting as a fluid-phase marker, this study revealed that, while macropinosomes do not contain invading *Shigella*, they are the major compartment present at the invasion site, in contrast to host endosomes that are excluded. Quantification of vesicle distribution in c-FIB/SEM ultrastructural datasets further supported this notion. FIB/SEM was instrumental to propose a bacterial entry mechanism based on an *in situ*-formed microenvironment around the pathogen that does not involve the subversion of major host endocytic pathways [15].

c-FIB/SEM was also used to image the enigmatic step of vacuolar rupture itself. Little is known about the biology driving this vital stage in the pathogen's life cycle. Strikingly, c-FIB/SEM revealed that during vacuolar rupture, macropinosomes and BCVs come into direct contact with each other, with ruptured membranes emanating into the cytosol from the macropinosome–BCV contact point. Live imaging and functional studies demonstrated that the involvement of macropinosomes in vacuolar rupture is dependent on specific bacterial effectors, including IpgD and IpgB1, and trafficking by the host machinery through the small GTPase Rab11, providing a major step forward in understanding this event. Overall, in this study c-FIB/SEM provided two key observations, one that stands in contrast to the prevalent paradigm (bacterial entry mechanism) and the other entirely novel (contact points at vacuolar rupture). By integrating these observations with live imaging, genetic and functional studies, a new model for early *S. flexneri* invasion into epithelial cells emerged that proposes two simultaneous processes through the entry of *Shigella* in a tight vacuole plus the formation of large macropinosomes in the vicinity that make contact, resulting in BCV destabilization.

Salmonella: A Novel Vision of the Differential Intracellular Niches

Salmonella enterica is a Gram-negative bacterial pathogen responsible for most cases of salmonellosis, a gastroenteritis that is usually self-resolving in healthy adults and is one of the most common causes of food-borne disease [39,40]. Infections are usually initiated by ingestion of contaminated food followed by bacterial adherence and invasion into the intestinal epithelium [41]. Like *Shigella*, *S. enterica* uses a type III secretion system to inject bacterial effectors directly into the host cytosol. These effectors induce membrane ruffling, promoting bacterial entry into the host cell via a macropinocytosis-like mechanism [37]. After internalization into a vacuole, *Salmonella* uses a second type III secretion system (T3SS-2) to further divert

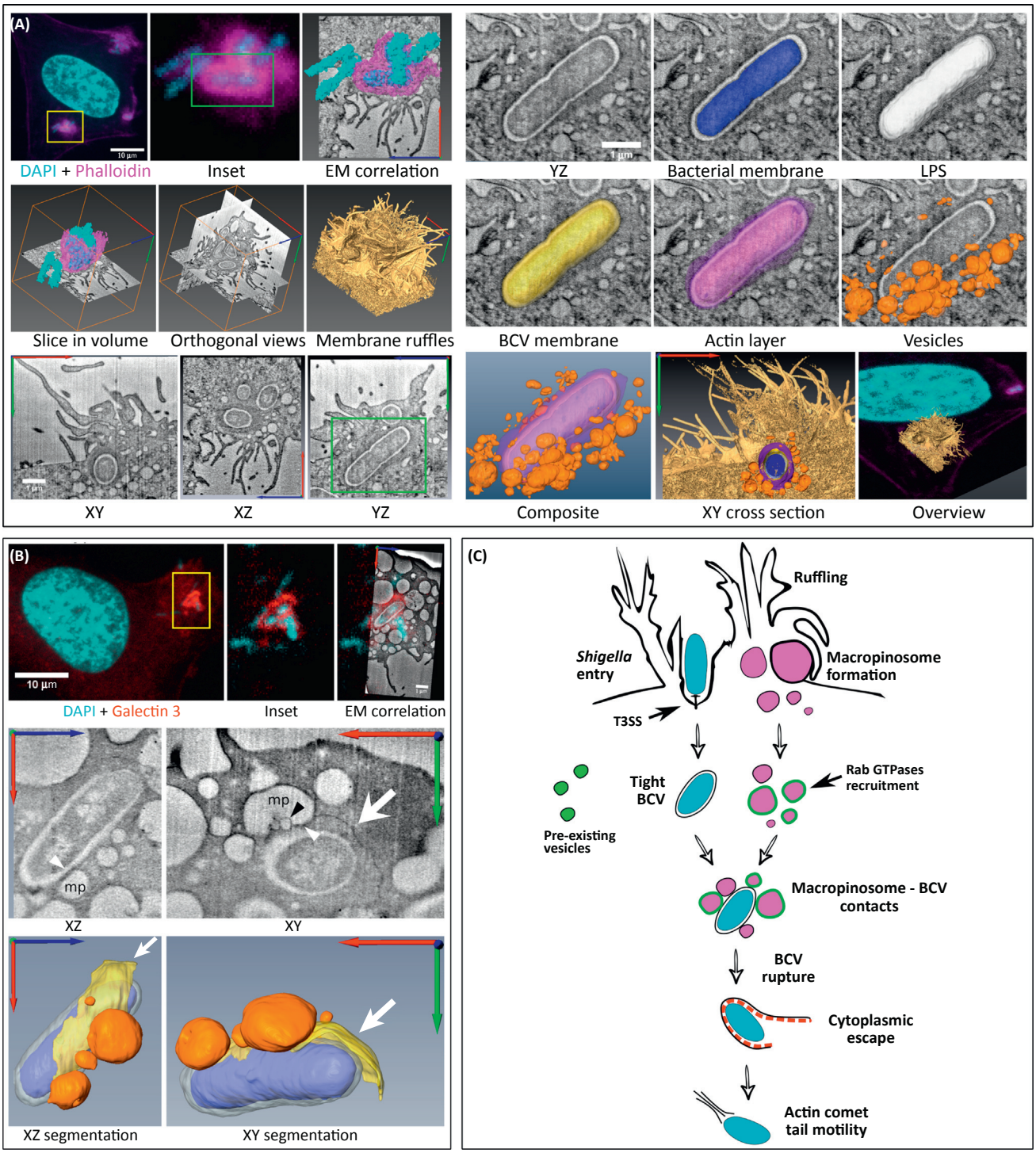

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Figure 2. Correlative FIB/SEM of *Shigella* Invasion. (A) c-FIB/SEM analysis of *Shigella* entry into epithelial cells. The *Shigella*-containing vacuole is identified by confocal microscopy via a surrounding cage of actin (bacteria in cyan, actin in pink). Careful segmentation of the FIB/SEM data identifies the bacterium (blue), its layer of lipopolysaccharide (LPS) (white), the vacuolar membrane (yellow), and the cage of actin (pink). The macropinosomes are segmented in orange. The lower right panel

(Figure legend continued on the bottom of the next page.)

host trafficking pathways [42]. During the course of infection, most bacteria reside within a modified phagolysosome called the *Salmonella* containing vacuole (SCV), while a minority (10–20%) escape into the host cytosol, where a subset begin to replicate rapidly, a phenomenon known as hyper-replication [43]. Many questions remain regarding the mechanisms of bacterial entry, the divergence of the pathogen into each scenario, SCV formation, hyper-replication or 'entrapment' by the autophagy machinery. Addressing these questions is particularly challenging as the underlying events are not only highly transient but some occur only in small subsets of the population. Using a similar approach to the one used to study of the conceptually simpler *Shigella* infection [44], we have addressed open questions relating to events during early *Salmonella* infection [45,46] (Figure 3). Once again, c-FIB/SEM was used for the identification of transient and rare events based on specific fluorescent markers, followed by an ultrastructural investigation giving information about the structure and organization of each event.

Salmonella entry into epithelial cells is classically thought to occur by uptake into heterogeneous macropinocytic pockets formed as a result of membrane ruffling [38]. c-FIB/SEM imaging of very early *Salmonella* infection revealed that, after uptake, bacteria reside in a tight vacuole containing a single bacterium (sometimes in division) that is not stained by fluorescent dextran (a macropinosome marker). This tight vacuole, termed the *Salmonella*-containing compartment (SCC), is distinct from the surrounding dextran-labeled macropinosomes of heterogonous size, strongly corresponding with our observations on *Shigella* entry. Using end-point and live imaging, the fate of the SCC was studied in both scenarios: SCV formation and hyper-replication. Within an hour postinfection, the majority of bacteria were observed inside spacious vacuoles positive for fluorescent dextran, suggesting that bacteria within the SCC have fused with macropinosomes to form a stable compartment. Such fusions determined the formation of the SCV. A minority of bacteria were never labeled with dextran, but were labeled by galectin-3-eGFP, a marker for membrane damage and rupture of the SCC. Half of them did not grow, which can be explained by their cytosolic targeting of the host autophagy machinery [47], while the other half grew rapidly. Together, these findings support the idea that the fate of the SCC (either fusion with macropinosomes or rupture) acts as the divergence point for the different intracellular niches formed by *Salmonella*. In accordance, c-FIB/SEM was performed on infection sites containing *Salmonella* labeled either with fluorescent dextran or galectin-3-eGFP. The former were found in spacious vacuoles (in contrast to the tight SCC) likely representing the SCV, further supporting the notion that the SCV is formed by SCC fusion with macropinosomes, while the latter were surrounded by a dissociating SCC, as indicated by a tight vacuole containing large gaps and pieces of membrane found in the host cytosol that could be directly correlated with the galectin-3 label. Strikingly, the dissociating SCCs were found in direct contact with macropinosomes, corresponding to observations made during *Shigella* vacuolar rupture. Overall, this work revealed common features of the earliest trafficking events of *Salmonella* and *Shigella* upon internalization, that is, the presence of two simultaneous processes at the invasion site: entry of bacteria into a tight vacuole (SCC for *Salmonella*, and BCV for *Shigella*) and macropinosome formation. Macropinosomes appear to be key for

correlates the segmented FIB/SEM dataset with the fluorescence image. (B) Vacuole rupture analysis by c-FIB/SEM. Galectin-3-mOrange is used as a fluorescence marker for vacuolar rupture (red). FIB/SEM of this region identifies contact points between macropinosomes (mp) and the rupturing vacuolar membrane peeling off the bacterium (white arrow). Segmented data are presented in the lower panels (blue: bacterium; orange: macropinosomes; yellow: rupturing bacterial containing vacuolar membrane). (C) A novel model of *Shigella* invasion of nonphagocytic epithelial cells. During the first step, *Shigella* (cyan) injects bacterial effectors to induce membrane ruffles at the site of contact with the host cell. This leads to the formation of macropinosomes (purple) and to the entry of *Shigella* into a tight vacuole (BCV) that is distinct from the surrounding macropinosomes. The newly formed macropinosomes act as targets for host Rab GTPases (green) recruitment, while host endosomes (light green), are excluded from the site of invasion. Finally, macropinosomes contact the *Shigella*-containing vacuole membrane (red) during vacuole rupture, leading to release of *Shigella* into the host cytosol. Reproduced from Weiner *et al.* [25].

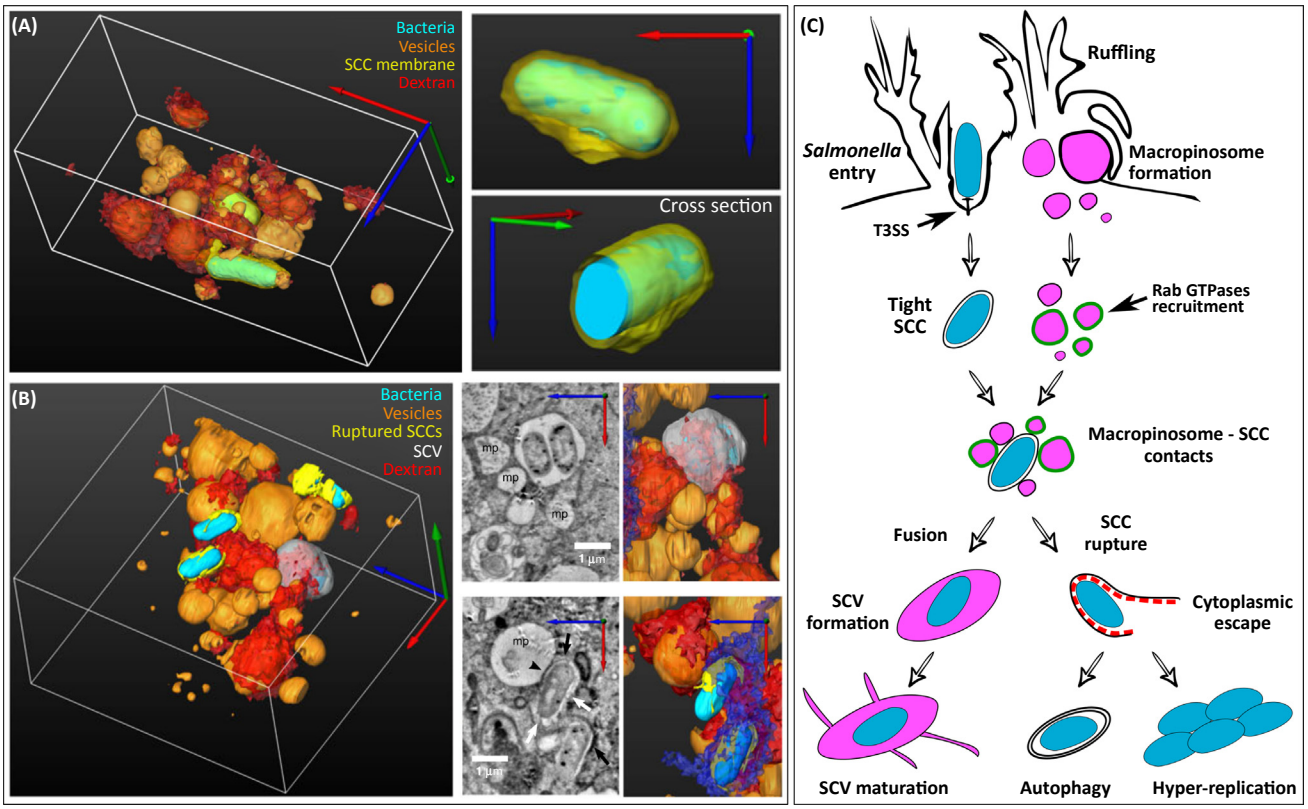

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Figure 3. FIB/SEM of *Salmonella* Invasion. (A) c-FIB/SEM analysis of early *Salmonella invasion.* Segmentation of FIB/SEM data shows that bacteria (cyan) reside inside the *Salmonella*-containing compartment (SCC) (yellow), a tight compartment distinct from surrounding macropinosomes (orange) labeled with fluorescent dextran (red). The tight vacuole surrounding invading bacteria at this stage is highlighted on the right. (B) c-FIB/SEM of infection sites containing *Salmonella* labeled either with fluorescent dextran (red) or galectin-3-eGFP (blue) (left). Segmentation of FIB/SEM data shows that bacteria (cyan) are found in two states: (i) inside a spacious vacuole (gray) labeled with fluorescent dextran, likely representing the *Salmonella*-containing vacuole (SCV) (upper right), and (ii) surrounded by dissociating SCC (yellow) labeled by galectin-3-eGFP (lower right). Dissociating membranes are in direct contact (black arrowhead) with surrounding macropinosomes (mp, orange). (C) A novel model of *Salmonella* invasion of nonphagocytic epithelial cells. *Salmonella* (cyan) injects bacterial effectors to induce membrane ruffles at the site of contact with the host cell. This leads to the simultaneous formation of macropinosomes (purple) and to the entry of *Salmonella* into a tight vacuole (SCC). The SCC and the surrounding macropinosomes are distinct. Then, the newly formed macropinosomes are targeted by host Rab GTPases (green), and direct contacts with the SCC membrane are formed. These interactions result in two possible outcomes: (i) macropinosome fusion with the SCC, leading to the SCV formation, followed by SCV maturation, and (ii) SCC rupture and bacterial cytoplasmic escape, followed by hyper-replication or entrapment by host autophagy machinery. Panels A and B reproduced, with permission, from Fredlund *et al.* [45].

determining the following step in the intracellular lifestyle of both pathogens, with macropinosome fusion with the SCC leading to the formation of the stable SCV, and macropinosome contacts with the SCC or BCV (without fusion) leading to SCC/BCV rupture and hyper-replication (*Salmonella*) or actin-based movements (*Shigella*).

c-FIB/SEM was also used by us to characterize the communication of the SCV with host cell compartments at later time points of infection [46]. This work has revealed direct contacts between the SCV and the host cell ER, as well as fusion between lysosome-like vesicles. A connection between phagosomes and the ER was proposed more than 10 years ago [48]; however, it has remained debated due to the limitations of biochemical fractionation procedures and the diffraction limit of fluorescence microscopy. Endomembranes belonging to the ER were identified by

staining against reticulin-4, followed by FIB/SEM and segmentation of the 3D ultrastructural datasets, revealing ER membrane sheets wrapping around the SCV, including structures that resembled membrane contact sites [46]. Furthermore, applying markers for host lysosomes, such as VAMP-7, revealed fusion events between the SCV and lysosomal-like vesicles. Overall, c-FIB/SEM allowed the direct differentiation of compartments that are closely juxtaposed, connected by membrane contact sites or fused with the SCV in the course of *Salmonella* infection.

Brucella: Unexpected Insights into the Replicative Compartment

Bacteria from the Gram-negative genus *Brucella* are the causative agents of brucellosis, a zoonotic disease that affects animals and humans [49]. *Brucella* is an intracellular pathogen primarily infecting professional phagocytes or dendritic cells at the onset of infection, where it can induce the formation of the *Brucella*-containing vacuole, initially associated with the endocytic pathway (eBCV) [49]. Vacuole acidification triggers the expression of a type IV secretion system that secrets bacterial effectors into the host cytosol, causing interference in lysosome fusion with the BCV. This allows vacuole maturation into a replicative *Brucella*-containing vacuole (rBCV) containing ER markers, where bacteria replicate to large numbers occupying a large part of the host cell [50]. The precise structure of this complex compartment, and how it relates to the ER and other host organelles, is difficult to decipher using light microscopy or two-dimensional electron microscopy, making it highly suitable for investigation by c-FIB/SEM.

Sedzicki *et al.* [51] used c-FIB/SEM in combination with structured illumination microscopy (SIM) to study the rBCV structure in detail (Figure 4). Using Emerald-Sec61 as a host ER marker, fluorescent bacteria were found to reside in a tight vacuole usually containing only a single bacterium. rBCVs contained links with each other and with ER cisternae, and when in proximity to the host nucleus with the outer nuclear membrane. Overall, rBCVs appeared to be integrated into the general cellular ER meshwork with continuous rBCV membranes stretching all the way to the outer nuclear membrane, suggesting that ER cisternae act as the replicative niche of *Brucella*. This niche could have the advantage of providing the pathogen with access to nutrients contained in the ER network, as well as to host mechanisms of membrane expansion required to maintain the growing rBCV.

In a related study, the interaction between the rBCV and mitochondria was investigated using 2D electron microscopy and light microscopy, showing that rBCVs and mitochondria may physically interact and that *Brucella* induces mitochondrial fragmentation in infected cells [52]. Mitochondria were also observed at close proximity to the rBCV by FIB/SEM [51], though their role in infection requires further analysis. Mitochondrial alterations have been described during infection by different pathogens, including *Listeria monocytogenes* or *Helicobacter pylori* [53,54]; nevertheless, it remains to be clearly demonstrated to which extent specific host processes are subverted by the different pathogens.

c-FIB/SEM: Future Perspectives and Challenges

Based on the examples above and several other studies (see Table 1), we propose that c-FIB/SEM is becoming a key technology for the study of intracellular pathogens at the cellular level. The subversion of the complex 3D architecture of infected cells, connectivity of compartments, the sites of pathogen action, the number and state of intracellular pathogens, and many other factors can be described and quantified using this technique. The combination of molecular specificity using fluorescence markers with volume ultrastructure has revealed precisely which host compartments are targeted by the invading pathogens, even if the events occur rarely and are highly transient.

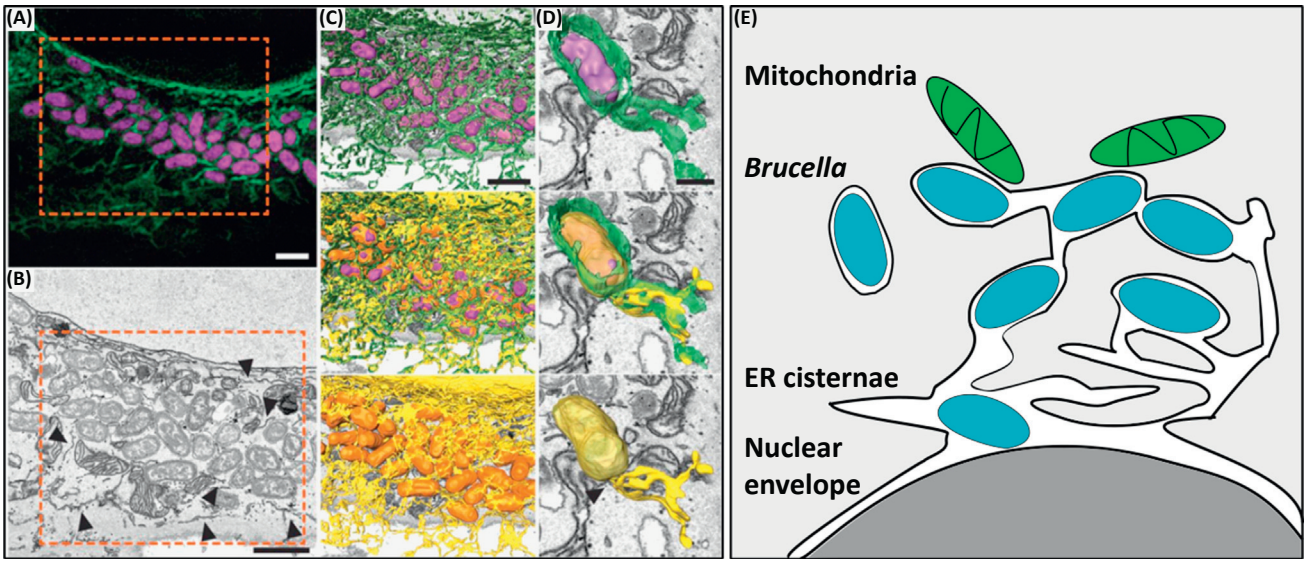

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Figure 4. Correlative FIB/SEM of the Replicative *Brucella*-containing Vacuole (rBCV). (A) Structured illumination imaging of the *Brucella*-containing vacuoles (rBCVs). *Brucella abortus* expressing deRed (magenta) is dividing within the endoplasmic reticulum (ER), labeled with Emerald–Sec61β (green). (B) A FIB/SEM slice showing flat cisternae between the rBCVs (arrowheads). (C) Segmentation of SIM data (top), FIB/SEM data (bottom) and overlay of the two datasets (middle). (D) A single rBCV is highlighted. rBCV membrane extensions are continuous with ER cisternae (arrowhead). Scale bars are 1.5 μm (A–C) and 500 nm (D). (E) A model for the localization of the rBCV and its interaction with host compartments. During infection internalized *Brucella* (blue) grows within the replicative niche, the rBCV. This niche can form a continuum with the ER and the nuclear envelope. In addition, *Brucella* is also found in isolated vacuoles, possibly representing another step of intracellular maturation. Furthermore, rBCVs are found in close proximity to mitochondria (green), though it is currently unknown if this is due to a specific interaction of the rBCVs with mitochondria or the result of crowding by the large number of intracellular *Brucella*. Panels A–D reproduced, with permission, from Sedzicki *et al.* [51].

Nevertheless, the application of c-FIB/SEM to the study of pathogens is still in its infancy (see Outstanding Questions). Establishing a rapid and efficient acquisition pipeline requires expertise in CLEM, sample preparation for electron microscopy, FIB/SEM acquisition and volume data image processing, segmentation, and quantification. In addition, precise knowledge of the studied infection model is needed to choose the appropriate markers and infection conditions. These combined expertise (and FIB/SEM technology itself) are not yet commonplace in most biological EM facilities, and routine biological FIB/SEM imaging (of pathogens and in general) is currently still undertaken in only a relatively small number of research facilities. Therefore, successful c-FIB/SEM projects on the host–pathogen cross-talk require efficient teamwork between biology experts and technologists. Once such a pipeline is put in place though, the transition from biological experiments to correlated and segmented data can be surprisingly quick and efficient [55] (Figure 1C). This pipeline can then be employed in tight coordination with other experiments done in the 'wet lab'. In this configuration, c-FIB/SEM has the potential to become an important driving tool in pathogen research, providing key experimental evidence that is subsequently confirmed by other techniques, as demonstrated in the examples above.

A limitation of the c-FIB/SEM approach presented here is the low degree of sample preservation obtained due to chemical fixation and sample dehydration performed using 'classic EM' preparation methods. As mentioned above, incorporation of high-pressure freezing and freeze substitution in c-FIB/SEM pipelines is possible, providing improved sample preservation. Recently, fully cryogenic FIB/SEM of samples preserved in a vitrified near-native state has been demonstrated, allowing imaging of large cellular volumes and even entire cells in pristine

conditions [56–58]. Cryo-FIB/SEM represents an exciting new development in the field that will likely be incorporated into CLEM and cryoET pipelines in the near future.

Another exciting direction is the use of super-resolution light microscopy in the c-FIB/SEM pipeline, enabling near matching of resolutions between fluorescent labels and ultrastructural information, providing precise localization of molecules of interest within the 3D volume [59]. Such localization can also be aided by protocols that preserve the fluorescent signal within the embedded sample so that light microscopy acquisition is performed after sample preparation for electron microscopy, thus avoiding movements and distortions that can occur in this process [60,61]. Another approach uses live imaging by fluorescence microscopy followed by fixation and c-FIB/SEM, tying in the dynamics of a pathogenic system with end-point correlated volume ultrastructural data [62]. Furthermore, c-FIB/SEM has been already achieved in the context of whole-body intravital imaging of single tumor cells, where it was also used in combination with microscopic X-ray computed tomography (microCT) [63,64]. Considering the relevance of small-animal models for infection research, this elegant pipeline allows the monitoring of pathogen–host interactions from the ultrastructural level to entire organs.

In-depth FIB/SEM data quantification represents one of the most exciting prospects of this technology. Traditional EM imaging of pathogens (particularly in their cellular environment) tends to be descriptive or semiquantitative, as features observed in sectional views or small three-dimensional cellular volumes are difficult to place within the larger cellular context. As FIB/SEM data provide equivalent resolution from all axes in large cellular volumes (or even entire cells), complex or convoluted features can be reliably identified, described in 3D, and placed within the overall architecture of the cell. Thus, quantifiable 3D parameters become available, such as the spatial distribution, surface area, volume and numbers of various compartments and organelles as well as their connectivity and relation to each other. In practice, the ability to extract quantitative information from acquired FIB/SEM data is still limited and often requires intensive manual examination and segmentation. Recent developments in image processing, particularly driven by ‘connectomics’ studies in neurobiology, have made great strides in improving automated segmentation and feature identification using machine learning [10,65,66]. As these developments become more widespread, FIB/SEM data analysis will become less labor intensive and technically demanding.

c-FIB/SEM is a meso-scale technique that lies at the boundary between light microscopy and high-resolution electron microscopy. This difficult to image ‘niche’ (sometimes referred to as the ‘resolution gap’ [67]) is of great importance for intracellular pathogen research, where the biological ‘theatre of events’ is a single cell, and understating the pathogen–host interface architecture is essential. c-FIB/SEM therefore is best used in complementation with other techniques, acting together to provide biological information across different scales and sample volumes. For example, dynamic light microscopy can be used to describe the temporal features of a pathogenic system at low resolution using specific fluorescent labels. c-FIB/SEM can then be employed to provide volume data on selected events via correlation. Finally, cryo-ET can be employed to provide fine structural details of a particular feature revealed to be of importance. c-FIB/SEM is becoming an indispensable tool for pathogen research as it can provide a wealth of biological information not obtainable by other technologies and is likely to facilitate many future discoveries in the field. Clearly, its applications range far beyond the study of pathogens, and its impact is likely to be felt in many other domains of biology as well.

Outstanding Questions

Will currently emerging volume EM techniques become widely accessible to biologists, in a similar manner to transmission electron microscopy (TEM) and scanning electron microscopy (SEM)?

Can c-FIB/SEM become a central tool for question-driven cell biology and pathogen research, hypothesis testing, and functional studies?

Will super-resolution light microscopy approaches be routinely integrated into c-FIB/SEM pipelines, providing near matching light and electron microscopy resolutions within the combined data sets?

What will be the impact of fully cryogenic FIB/SEM – a very recent development allowing expansive sample volumes to be studied at high-resolution in near-native conditions – on our understanding of cellular and tissue organization and pathogen–host interactions?

Can developments in the domain of automated image segmentation and machine learning eliminate entirely the need for manual segmentation of volume EM data?

FIB/SEM and other volume EM techniques can provide new types of quantitative information like the precise 3D localization, volume, shape, and connectivity of organelles and compartments within expansive cellular and multicellular environments. How will such quantitative information contribute to our understanding of cellular organization and pathogen–host interactions?

Can FIB/SEM be efficiently and routinely correlated with live cell imaging? Intravital imaging? Microscopic X-ray computed tomography (microCT)? Electron cryo-tomography?

How will the major advances made in recent years in the domains of volume EM, cryo EM, correlative microscopy, and super-resolution light microscopy contribute to our fundamental understanding of the structure and organization of cells and tissues?

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