doi:10.1017/S1431927616001896
Microsc. Microanal. 22 (Suppl 3), 2016

The Agony and the Ecstasy: Correlative Microscopy from Photons to Electrons and X-Rays. Lessons from Recent Case Studies.

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Integration of different microscopy modalities combines the strengths of various technologies and thereby paves the way for improved resolution, context and understanding of events in living systems [1]. With the submicrometer scale resolution of fluorescence microscopy (FM), cellular dynamics are revealed, but the nanometer scale biological context enabled by electron microcopy (EM), is lost. Correlative Light and Electron Microscopy (CLEM) therefore links functional information from fluorescent protein markers with their ultrastructural localization in cells, and by adding micro X-ray Fluorescence (XRF) imaging, further quantitative analytical data can be integrated to clarify metabolism, biogenesis and cell-cell interactions. Where light microscopy can reveal live cell events, electron microscopy requires that biological samples should be fixed and either embedded in resin or dried. Localization of structures depends on the ability to enhance their contrast with heavy metals for both scanning (SEM) and transmission electron microscopy (TEM) or tagging the biomolecule with a functionalized metal particle. Live cell imaging is therefore impossible using EM, and rare events can only be appreciated if large volumes of the sample can be visualized at high lateral and axial resolution. Such technologies are available through Serial Section-SEM [2] and Serial Block sFace (SBF)-SEM [3]. For integrated CLEM [4,5], where more than one microscope modality need to be visualized simultaneously, or where sample preparation is aimed at the final (EM) imaging modality, processing must be optimized (i) to retain the fluorescent signal and (ii) to preserve ultrastructure for EM localization and correlation. Genetically encoded probes for pre-embedding labeling (mini-SOG, APEX2) additionally enables EM localization of targeted molecules and CLEM [6]. For sequential data acquisition and matching of regions of interest (ROIs), fluorescence need not be preserved, since the sample is processed for EM after FM imaging. However, it is imperative that consecutive steps of sample processing should preserve chemical and physical properties of structures to fit the requirements of the proceeding modality, and additionally preserve the relative positions in a navigation map of ROIs.

In medical sciences various microscopy modalities are used to explore and define aspects of cell and tissue development, stem cell morphogenesis, bacterial infection and neuronal growth. Recent case studies in consecutive data acquisition for CLEM include (i) Localization and characterization of GFP positive hair (stereocilia)-like cells derived from cochlear progenitor cells grown on chicken feeder cells (Figure 1A) [7], (ii) Cell wall dynamics in *Staphylococcus aureus* cytokinesis (Figure 1B) [8], (iii) Localization and regulation of actin enrichment in dendritic filopodia during neural development [9], (iv) Identification of polarized cadherin fingers that collectively guide endothelial cell migration and tissue development from individual cells; (v) The role of micronutrients in *Helicobacter pylori* infection of host epithelial cells through injection of cagA effector protein (Figure 1C); (vi) Investigation of the effect of CLARITY techniques [10] on ultrastructural features of the hippocampus.

Various substrates for cell culture have been developed to enable precise localization from one modality to the next. Substrates need to (i) preserve fiducials through various imaging modalities and sample processing, (ii) be light-transmitting, (iii) functionalized for cell growth and retention of fixed cells, (iv) compatible with reagents and instruments used for SEM processing, while (v) also being either

conductive or allow conductive coating for extended scan cycles. Where experimental requirements are incompatible with commercial navigation substrates, customized substrates may need to be designed through photolithography, metal coating of a masked grid, mechanical marking, or overlaying of beam-compatible 'finder grids' onto cell sheets. Transfer and processing of samples between microscopy modalities require additional care to retain ROIs in their mapped position, preserve ultrastructure and prevent artifacts and contaminants on the specimen surface.

Where X-ray analysis follows SEM, generic procedures introducing heavy metals (OsO4; Au-Pd) should be avoided, and alternative techniques such as Variable Pressure-SEM must be explored. Localization of ROIs from SEM to X-ray may be accomplished through tagging of an organism or cell type, e.g. immunogold localization of bacteria on their host cells (Figure 1C). For CLARITY brain samples, where heavy metal staining is compromised through the loss of lipids from cell membranes, EM correlation with FM is additionally impacted by poor signal to noise ratios. High sensitivity APEX2 provides a novel solution for CLEM in CLARITY tissue, where proteins are preserved despite the loss of lipids. Automated navigation and imaging between different modalities is rapidly evolving to decrease the extensive data acquisition times generally required in correlative microscopies.

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[11] The author thanks Drs M Amieva, A Hayer, X Zhou, S Sinkkonen and A Tom for collaboration and contributions to this work. CSIF Beckman Center, Stanford University, is thanked for financial support.

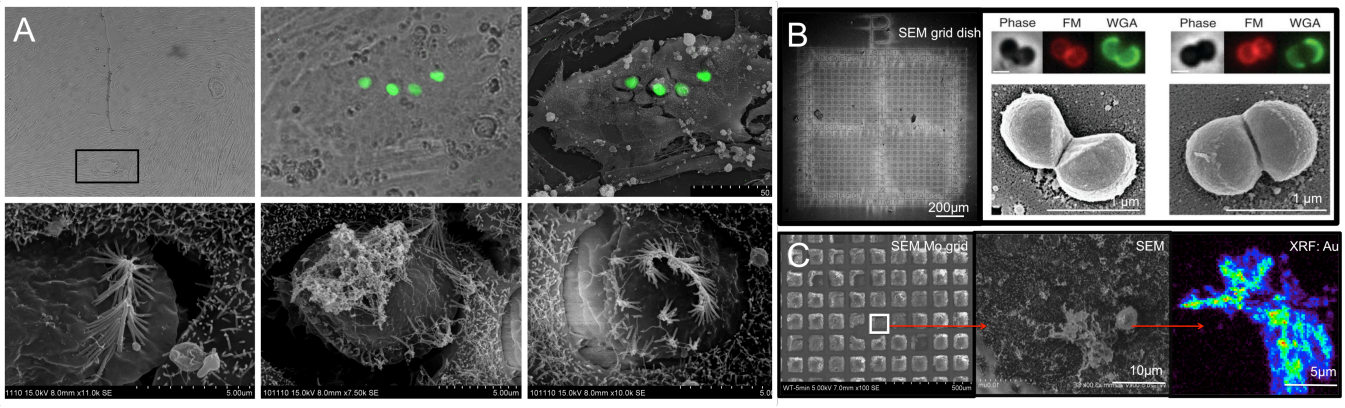

Figure 1. CLEM through consecutive data acquisition from LM to SEM (A, B), and SEM to XRF (C). (A): Morphology of GFP-positive hair-like cells; (B): *S. aureus* cell division on relocation culture dish; (C): Localization of *H.pylori* on MDCK cells using Molybdenum grids and immunogold labeling.

https://doi.org/10.1017/S1431927616001896 Published online by Cambridge University Press