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It's a Small, Small World: A Brief History of Biological Correlative Microscopy

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1.1 It All Began with Photons

Light microscopy (LM) is arguably the oldest technology still used in scientific research today. Until the mid-1600s, the world of structures smaller than about 400 microns was unseen and unknown. While the principles of using lenses to magnify were known as far back as Euclid (c. 300 BCE) [1], microscopy had to await technical developments in the manufacture of lenses and the casings to hold and position them, before they could be used to extend the power of human visual resolution. The earliest published description of a biological sample viewed using a simple one lens microscope was probably in 1658's *Scrutinium pestis physico-medicum* [2] written by a German friar Athanasius Kircher. In this manuscript he describes the presence of "little worms" in blood that he associates with disease; thus anticipating the germ theory by almost 100 years.

Around the same period, Dutchman Antonie van Leeuwenhoek used his single-lens microscope to examine samples of mold, bees, and lice, and reported these and other observations to the Royal Society in a series of letters beginning in 1673. It was when he went on to look at samples of blood, tooth plaque, and sperm that he observed that individual small structures that moved of their own volition! When he reported his observations in a letter to the Royal Society in London in 1676, they were met with great skepticism. In 1677, a delegation was sent to determine if he was brilliant or demented. Having vindicated his observations, he was elected to the Royal Society in 1680. However, while the best of van Leeuwenhoek's microscopes had an impressive maximum magnification of 260 times, their resolving power was limited to about 1.4 μm [3].

Although simple one-lens microscopes like Van Leeuwenhoek's were impressive, a Dutch inventor by the name of Cornelius Jacobszoon Drebbel brought a new device to London [4] even earlier (1619), a two-lens microscope that possessed higher magnification capacity than the Van Leeuwenhoek instrument since it was based on the principle that in a two-lens microscope the total magnification of the lenses was multiplicative [5]; although the resolution was limited by optical aberrations.

Using a microscope very much like Drebbel's, but with an improved source of illumination, the Englishman Robert Hooke was able to see details in pieces of plants, animals, and insects that had previously been unknown. For example, he observed that a piece of cork bark was composed of many small rectangular compartments. They reminded him of the small rooms that monks slept in. He called them cells, a name we still use today; had he called them chambers we might be studying chamber biology instead. He published these observations as well as the first recorded attempt to make measurements using a microscope in his 1665 book *Micrographia* [6]. These early studies of the invisible world of cells represent the birth of modern microscopy.

In the eighteenth and nineteenth centuries, microscopes became progressively more powerful, lens design was improved to remove aberrations, and innovations such as the use of polarized illumination were introduced. In the 1880s the German scientists Ernst Abbe and August Valentin Köhler working with Carl Zeiss brought together a sophisticated lens design [7] and improved illumination methods [8, 9] to create microscopes that could resolve subcellular structures. Abbe was the first to mathematically calculate the limits of microscope resolution using photons [10]. His calculations showed that the wavelength of visible light and the angle from which the diffracted light is collected defined the limits for microscopic resolution. Thus, the Abbe diffraction barrier of 188 nm was elucidated, and this would remain the limit of light microscopy until the advent of super-resolution techniques some 125 years later.

1.2 The Electron Takes Its Place

In the 1920s, while light microscopy still had to fully exploit its resolution possibilities, a young French physics student was pondering the theories of Einstein, in particular the nature of electrons, and wondering if they had a wavelength. His name was Louis de Broglie and the equation describing the wave nature of electrons was at the heart of his PhD thesis [11]. In a triumph of early career achievement his thesis secured him the 1929 Nobel prize in physics! Being a theoretician, he had no practical use for his work and went on to the next equation. Fortunately, there were more practically minded physicists who did see the use of the wave nature of electrons. Ladislaus Marton in Brussels, and Ernst Ruska, Max Knoll, and Ernst Brüche in Berlin developed simultaneous prototype transmission electron microscopes, which proved that not only did electrons have a wavelength but also that they could be focused by electromagnetic lenses and used in the same manner as light was used in optical microscopy [12]. Ruska theorized that under the right conditions these microscopes could achieve a resolution of 2Å, which was proved correct almost 40 years later [13].

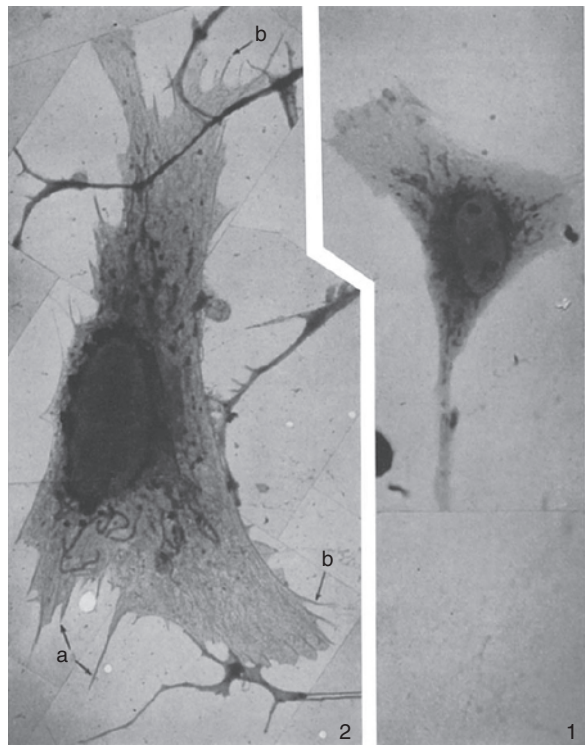
Biologists rejoiced at the news that smaller subcellular structures could finally be resolved; however, it came at a price. Specimens had to be imaged in high vacuum and radiation damage from the strong electron beam was intense. Despite that, Marton published the first biological electron micrograph of a sample of *Drosera intermedia*, sundew, in the journal *Nature* in 1934 [14]. While this was a breakthrough, the actual resolution of electron micrographs would be insufficient to produce useful scientific data for another 20 years. So until almost the 1960s, electron microscopes were like the optical microscopes of the seventeenth century, largely curiosities.

1.3 Putting It Together, 1960s to 1980s

Although both light and electron microscopy continued to improve, it wasn't until the 1960s that researchers tried to combine the two imaging techniques. When searching the early literature for correlative microscopy publications, it becomes obvious that the term as we now use it, to indicate light and electron microscopic studies on the *same* area of the *same* sample, has evolved over time. The earliest references are frequently studies of the same tissue or sample type but not necessarily on the same specimen; thus, they are more comparative than truly correlative. The earliest paper that we have found that imaged a sample in a light microscope with a similarly prepared sample in an electron microscope is from the pioneering work of Keith Porter, where chick embryonic fibroblasts were cultured on a formvar substrate, fixed and imaged (Figure 1.1) [15]. This was only done as a proof of principle for developing EM techniques, though, and no attempt was made to draw conclusions from any correlation. A correlative study from 1960 by Goodman and Morgan was performed on separate cell cultures and published as two papers, one for light [16] and one for transmission electron microscopy (TEM)[17].

Other correlative studies from 1969 [18] and 1970 [19, 20] used biopsy samples that had been divided and processed for either light or electron microscopy, and then extrapolated between the morphological findings in each. Additional studies of correlative microscopy went a step further and used the same sample but adjacent sections. In

Figure 1.1 The first micrograph to compare a sample imaged with a light microscope; 1) and an electron microscope; 2), was published by Keith Porter in 1945[15]. While not truly correlative, e.g. of the same specimen, this did demonstrate that samples prepared with the same procedures could be imaged using multiple methods. Reproduced with permission of ROCKEFELLER UNIVERSITY PRESS via Copyright Clearance Center ©1945.



1970, Watari and coworkers published a study of the islets of Langerhans using adjacent resin-embedded sections [21], and in 1979, Hyde et al. used the same block to first cut thick sections and inspect them by LM, then selected areas were cut out from these samples, and thin sectioned for TEM [22]. A very early attempt to combine immunohistochemistry with TEM was published in 1974 by Bordi and Bussolati [23].

In 1980, Gonda and Hsu combined LM, scanning electron microscopy (SEM) and TEM to study developing mouse blastocysts [24]. These early studies, although not meeting the criteria for correlative microscopy that we use today, were examples of researchers trying to use multiple microscopy methods to bridge the resolution gap between photons and electrons.

It was probably the 1967 article by McDonald, Pease, and Hayes [25] that examined sectioned rabbit tissues by LM and SEM, that marks the first use of correlative microscopy with the specific purpose of adding the extra resolution available in the EM to the LM data (Figure 1.2). A 1969 paper by McDonald and Hayes used fixed, dried blood cells and clots and correlated images of the same cells using their morphology and proximity to neighboring cells to identify them [26]. In 1971, a short technical note was published by Ayres, Allen, and Williams using specimens from a cervical biopsy that were inspected by SEM, then reprocessed for LM imaging [27]. At the same time, a group led by H.D. Geissinger at the University of Guelph was working intensely on correlative microscopy and matching the same area in cell preparations and tissue slices using SEM and LM [28]. In a 1973 paper, Geissinger, Basrur, and Yamashiro constructed a custom-built holder with a measuring caliper that could transfer between the LM stage and SEM specimen chamber [29,30], and by use of a method of correlated integers [31] they were able to reacquire the same coordinates and image the same area. Geissinger, Abandowitz, and Josefowicz used the same technique to examine hair shafts in transmitted and reflected light and SEM [32].

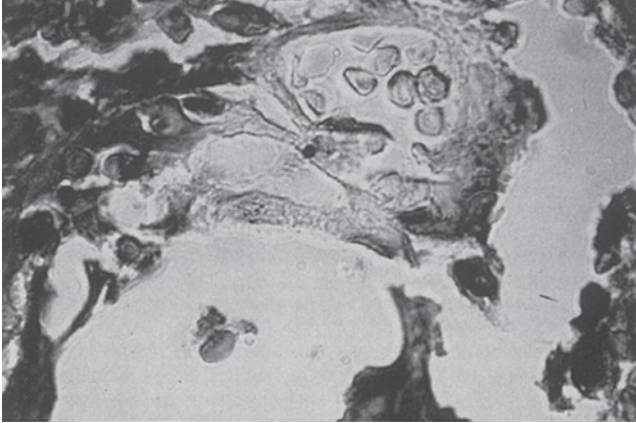
Geissinger and coworkers continued to explore the possibilities of correlative microscopy, combining many imaging modalities: SEM-interferometry [33], and LM polarization-SEM-TEM (Figure 1.3) [34]. This approach of combining multiple LM and EM techniques was also adopted by other investigators. A paper from 1989 used a combination of live cell video microscopy, low-voltage SEM, and high-voltage TEM to study membrane associated glycoproteins in human platelet cells [35], and that same year a paper used intravital video microscopy, LM and TEM to study capillary growth [36].

These early efforts to bridge the resolution scale were pioneering and led to a greater interest in using combined microscopic techniques to increase the data content of bio-imaging experiments. This interest was further demonstrated in 1987 with the publication of the first book describing CLEM instrumentation and methods [37].

1.4 CLEM Matures as a Scientific Tool 1990 to 2017

In the next 25 years, the technique would continue to progress, not only with specific CLEM developments aimed at more precise and faster correlation but also by constantly implementing improvements made in the LM and EM fields. For example, in the early 1980s LM in the life sciences was reinvigorated by the development and commercialization of confocal laser scanning microscopy [38, 39]. This was quickly supplemented by the discovery of green fluorescent protein (GFP) [40, 41] and other

(a)



(b)

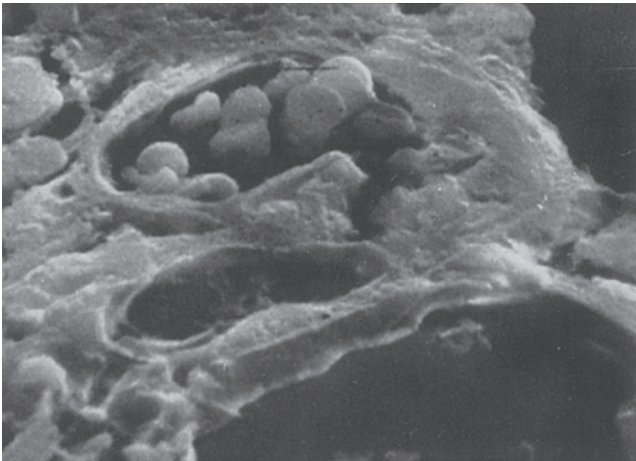


Figure 1.2 Quite possibly the first truly correlative micrograph, i.e., of the same sample imaged in both the LM (a) and EM (b), published by McDonald, Pierce and Hayes in 1967 [25]. An “area of delayed radiation lesion of rabbit sensory cortex” in a 4 μm section of paraffin embedded tissue. The authors used the correlation to point out features in the EM that were not obvious in the LM. Reprinted by permission of the publisher, Springer Nature, from laboratory Investigation © 1967.

fluorophores, allowing functional imaging in live cells [42, 43]. In the 1990s, subdiffraction-limited or super-resolution LM techniques with resolutions below the limit set by Abbe’s law started to appear. First was stimulated emission depletion (STED) microscopy [44], followed by structured illumination microscopy (SIM) [45], stochastic optical reconstruction microscopy (STORM) [46], and photoactivated localization microscopy (PALM) [47] More recent developments such as light sheet microscopy [48], tissue clearing, expansion microscopy [49], and adaptive optics [50] continue to extend the capabilities of LM and in due course will find their way into CLEM.

On the EM side, electron tomography (ET), which offers 3D visualization of a selected part of a specimen at very high resolution, has been continuously improved since 1968

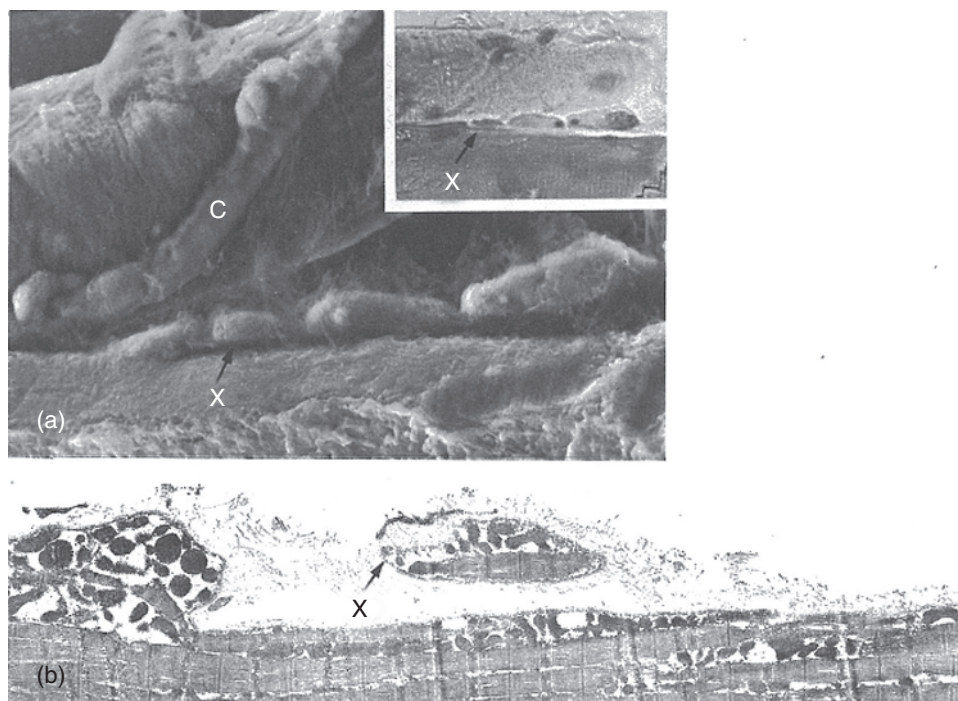


Figure 1.3 The laboratory of Professor H.D. Geissigner pioneered the development of different correlative microscopy workflows using multiple imaging modalities. This elegant example from a 1980 paper demonstrates the extra information to be gained through the correlation of LM (inset $\times 400$), SEM ($a \times 1500$) and TEM ($b \times 5400$) examining a sample of human muscle in a patient with muscular dystrophy. Reprinted by permission of the publisher from *Ultrastructural Pathology* © 1980 (Taylor & Francis Ltd, <http://www.tandfonline.com>).

and with the growing computational power is still increasingly applied and optimized. One of the most recent developments is ET done under cryo-conditions [51, 52], providing exciting new applications in the field of structural biology. It is also of great importance for CLEM that the resolution of SEMs has been steadily improved, now almost approaching the TEM level [53]. Moreover, SEMs gained extended automated capabilities for 3D imaging [54, 55, 56], which greatly facilitates CLEM in the z -axis. Further improvements in LM and EM have made them more complementary in terms of resolution, contrast generation, and image dimensions. The promising power of combining these complementary modalities in integrated or modular microscopy settings has boosted the development and applications of CLEM in the last decade.

One of the main challenges in CLEM studies is to use LM to determine where you are in the landscape of the nanoworld of EM. Sadly for microscopists, a cellular version of GPS does not yet exist. To correlate a region of interest (ROI) in a light micrograph to the corresponding area in an electron micrograph is no easy task. Ideally, what is needed is a probe that can be easily visualized in both microscopes. An early but noncorrelative immunohistochemical study did develop such a probe using horseradish peroxidase (HRP)-antiperoxidase-diaminobenzidine (DAB), but only inspected it in the TEM [57].

To the best of our knowledge, the first demonstration of a directly correlative tracer, visible in both LM and EM, was in 1980 when Roth synthesized a FITC-protein A-colloidal gold complex to label antibodies to amylase in sections of pancreas [58]. In 1982, Maranto used photoconversion of the fluorescent dye Lucifer yellow in the presence of DAB to create an osmiophilic polymer [59], and this was followed by other studies using photooxidized fluorophores [60, 61]. In 1987, Quattrocchi and colleagues synthesized fluorescent nanospheres linked by IgG to protein A-colloidal gold, and used them for retrograde neuronal labeling [62]. Polishchuk in 2000 used live-cell confocal microscopy to localize GFP to the Golgi complex and subsequent HRP-DAB reaction to relocate the ROI by serial section TEM to create 3D reconstructions [63]. In 2001, Adams reported the engineering of ReAsh-EDT₂ [64] a biarsenical ligand that could photooxidize tetracycline tagged GFP for use in CLEM studies [65]. In 2005, Grabenbauer demonstrated a new method that proved it was possible to use GFP to directly photooxidize DAB, thus allowing for the correlative visualization of endogenously expressed proteins [66]. This was followed in 2011 by the engineering of Mini-SOG, a fluorescent flavoprotein that was both fluorescent and a high-efficiency photooxidizer developed specifically by Roger Tsien for CLEM [67]. All these attempts were hampered by the diffusible, nonquantitative HRP DAB reaction product that decreased the precision of the correlation.

In 2012, Martell and co-workers developed APEX, a small (28-kDa) genetically engineered peroxidase that can be coupled to fluoroproteins, remains active following fixation, creates a more precise localized reaction and does not require light to reduce DAB [68]. APEX is now also available in a modular form incorporating a GFP binding protein [69]. In 2015, the laboratory of Ben Giepmans developed a probe called FLIPPER, expressly for CLEM studies containing a fluoroprotein and a peroxidase that could be genetically expressed [70]. Most recently Arnold et al. developed a cryo-LM stage that, combined with fiducial markers and a computational algorithm, is able to allow for precise correlation between cryo-LM and FIB-SEM [71] (Figure 1.4). This development is very exciting, as CLEM can now be used to precisely guide the FIB milling process of vitrified cellular samples and capture specific structures in their native orientation.

Since the interest in CLEM continues to increase, other types of probes are becoming rapidly available. In parallel and addition to the DAB-dependent probes already described, others tried to preserve fluorescent signals in resin-embedded tissues to make the CLEM workflow more flexible. In 2014, Peddie et al. succeeded in retaining fluoroprotein signals in resin embedded heavy metal stained tissues [72], and in the same year, Perkovic and co-authors published a similar method for organic fluorophores [73]. Super-resolution probes for CLEM have also been developed. In 2015, Paez Sengla and colleagues developed fixation-resistant photoactivatable fluoroproteins for CLEM [74]. Recently, scientists working in Jena, Germany, synthesized polylactide nanoparticles incorporating iridium (III) complexes [75], Müller et al. reported the use of self-labeling protein tags for time resolved CLEM experiments [76] and the laboratory of Roger Tsien described the use of Click-EM for imaging metabolically tagged non-protein biomolecules [77]. Nonfluorescent detection techniques to relocate ROIs have also been investigated. Physical marking of tissues using an infrared laser can help to reacquire an ROI at the EM level [78]. In 2012, Glenn and co-authors developed probes that are cathodoluminescent, raising the possibility of discriminating between multiple probes at the EM level [79]. In 2015, Nagayama et al. demonstrated eGFP cathodoluminescence [80], and

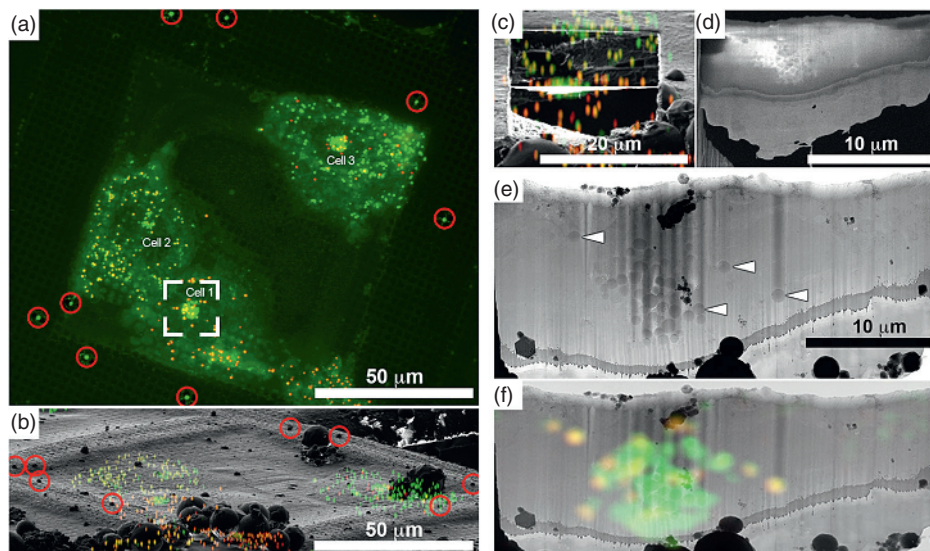


Figure 1.4 Correlation approaches between cryo-LM and FIB-SEM is very promising to precisely guide the FIB milling process of vitrified biological samples, and paves the way for visualization of single molecules in their native *in-situ* states. Arnold et al. developed a cryo stage for spinning-disk microscopy at cryogenic temperatures, and employed this approach to guide the FIB milling process to capture fluorescently labeled lipid droplets, in 300 nm lamellas. Reprinted by permission from: Arnold, Jan, et al. "Site-specific cryo-focused ion beam sample preparation guided by 3D correlative microscopy." *Biophysical Journal* 110.4; 2016): 860–869.

Furukawa and co-workers developed rare-earth nanophosphors for cathodoluminescent imaging in scanning-transmission EM [81]. In 2016, Fukushima and co-workers reported the development of yttrium oxide nanophosphors, which are both fluorescent and cathodoluminescent as well as electron dense [82] and Hemelaar et al. reported the use of fluorescent/cathodoluminescent nanodiamonds for correlative studies [83].

Although we still lack a cellular equivalent of GPS, a 2017 publication, integrating several of the developments mentioned above, has demonstrated a correlation technique using cathodoluminescence with a precision of <5 nm between LM and EM images [84]. While the development of probes is invaluable for the progress of CLEM, their applications are maximized thanks to the many computer vision and bioinformatics specialists who have made strides in the "back end" of the process; that of data analysis and eventual overlaying of the LM and EM digital data [85, 86, 87, 88]. With the development of specifically engineered probes and software, the CLEM workflow has become easier and more accessible to nonspecialist labs.

A way to minimize the effort to find back ROIs by LM and EM is to combine LM and EM in one microscope. The first attempt at such an integrated device was in 1978, when Hartmann and co-workers published a note describing an attachment to a commercial SEM that incorporated light microscope optics [89]. Further adaptations were made, and in 1981 JS Ploem presented a prototype Leitz instrument at the VIII Conference on Analytical Cytology and Cytometry [90]. In 1982, Wouters and Koerten also published an integrated instrument for LM and SEM [91]. Then, for over 20 years there was little progress on the instrumentation front, until in 2008 three groups from Utrecht

University and Leiden University Medical Center collaborated on the design of an adaptation to a TEM that incorporated a tilting sample holder and an aspherical (NA 0.55) objective lens mounted inside a TEM column perpendicular to the electron beam and connected externally to a scanning confocal microscope [92]. This integrated light and TEM was commercialized by FEI (now Thermo Fischer) under the name iCorr.

Although this approach worked successfully, it required special specimen preparation that had to compromise between preserving the fluorescent signal and maintaining ultrastructural integrity and contrast, which for room-temperature CLEM still is a challenge. An improvement on this design introducing cryo-EM to observe fluorochromes in their hydrated state was published in 2012 [93].

The following year a collaborative effort between groups from both academia and industry published the design for an integrated room-temperature fluorescence-SEM. This system employed a reflective mirror and a 45X NA 0.41 objective lens placed in an SEM column in which holes were bored to allow passage of the electron beam [94]. This design also required special specimen preparation with fixation in low-concentration gluteraldehyde and dehydration insensitive fluorochromes. As far as can be determined, this design was never commercialized.

In 2010, Nishiyama and co-workers published the design for a combined instrument in which a fluorescence microscope was mounted over an inverted SEM with the sample in a chamber constructed of a silicon nitride film [95]. This was later marketed by JEOL as the ClairScope. Because of its limited magnification and resolution capacity, it was never widely adopted but proved the impetus for other designs of integrated instruments.

In 2011, Albert Polman in AMOLF, Amsterdam developed the SPARC, a custom-built stage that could be installed in an SEM containing a swinging parabolic mirror connected to an external CCD camera recording cathodoluminescence [96]. This design was used primarily for materials science and nanophotonics applications, although recent breakthroughs in biological applications could change that [97]. In 2013, the Charged Particle Optics group of Delft University of Technology developed SECOM, which included a high NA optical lens inside the vacuum chamber directly below the SEM pole piece [98, 99]. Both SPARC and SECOM are marketed by a spinoff company, DELMIC BV. In 2013, DELMIC joined with Phenom-World BV to develop the Delphi that used a similar design to SECOM but as a standalone instrument; it was unveiled in 2014 at the IMC in Prague. A SECOM variant including super-resolution capability was released in 2016. Using this SECOM platform, Peddie et al. recently presented strong stable blinking properties of GFP and YFP *in-vacuo*, and used this to achieve super resolution CLEM of resin-embedded cells [100] (Figure 1.5). Additional developmental work is underway on integrated instrumentation such as the inclusion of a miniature fluorescence microscope into a serial blockface sectioning SEM system for 3D CLEM studies [101]; and with the growing interest in correlative microscopy we can expect to see new instruments with additional capabilities that could bring CLEM into more widespread use.

The early trend to combine multiple modes of light and electron microscopy continued into the 1990s with various permutations of light, fluorescence, video, SEM, and TEM being employed [102], and increasing numbers of studies that went beyond mere proof of principle to address significant scientific questions [103, 104, 105]. Newly developing microscopic techniques were integrated into CLEM studies including: intravital

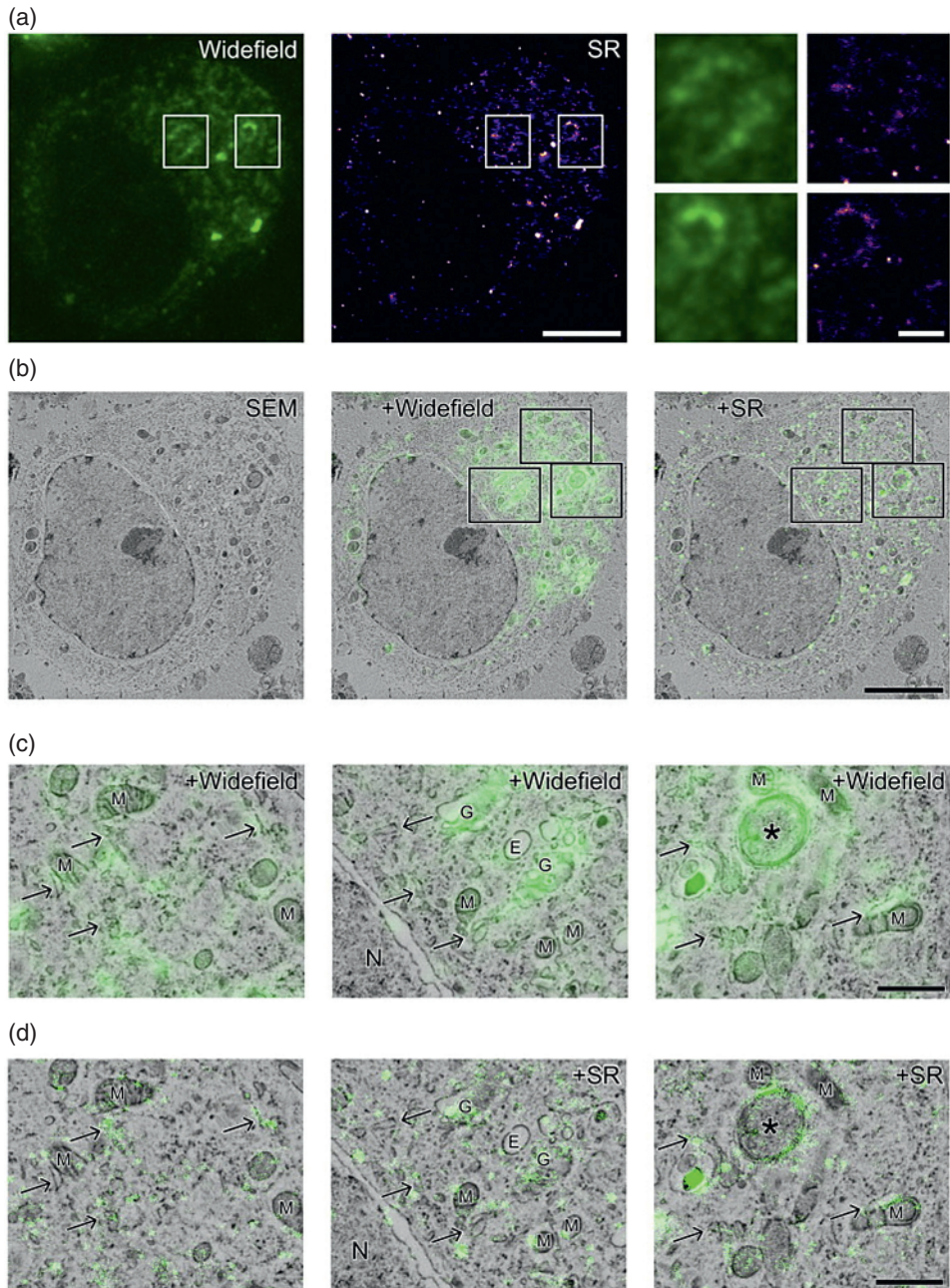


Figure 1.5 Correlative single molecule localization microscopy and EM of resin embedded cells in an integrated super-resolution LM in SEM. This approach successfully localizes the lipid DAG to subdomains of Golgi membranes, endoplasmic reticulum, cristae and outer mitochondrial membrane (c and d). Reprinted from Peddie, Christopher J, et al. "Correlative super-resolution fluorescence and electron microscopy using conventional fluorescent proteins *in vacuo*." *Journal of Structural Biology* 199.2: 120–131; 2017) under creative commons license 4.0.

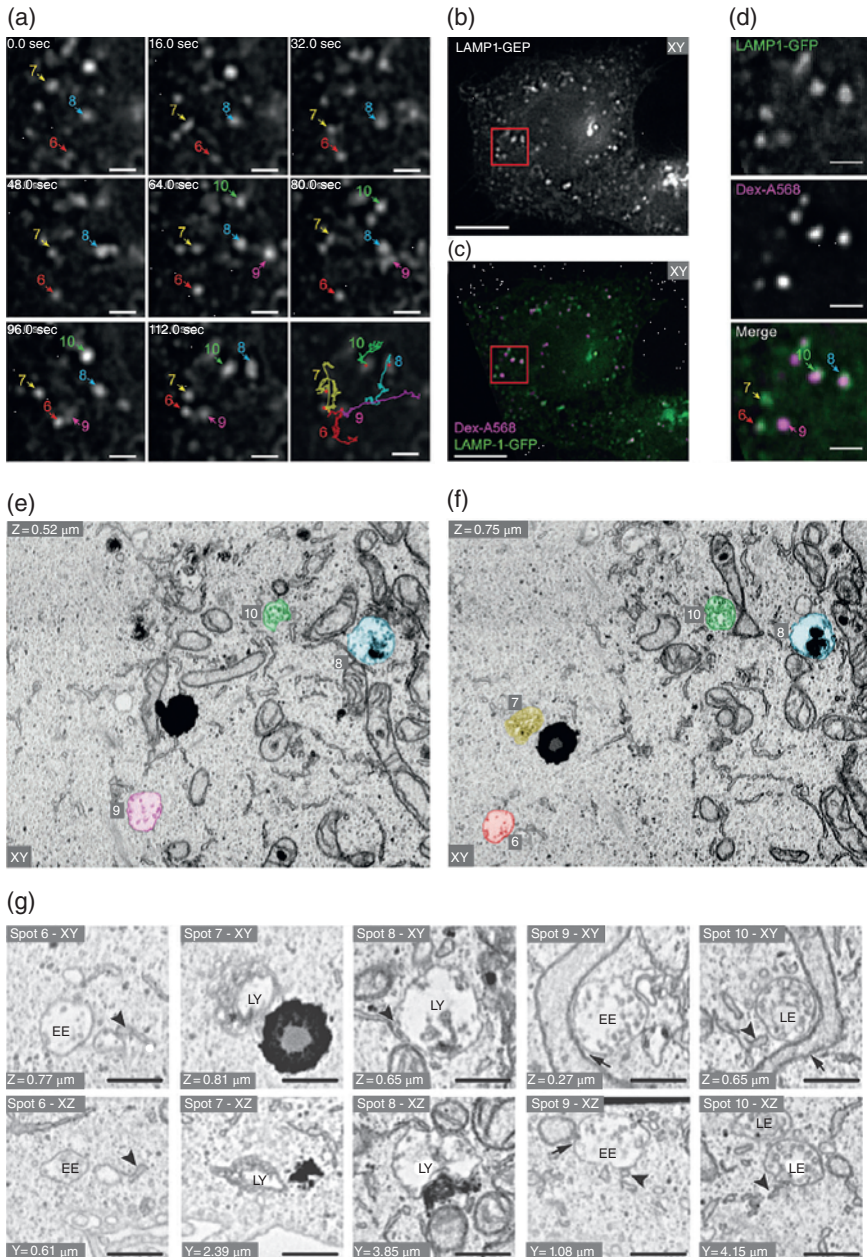


Figure 1.6 Correlation of live-cell imaging to 3D-EM integrates multiple dynamic and ultrastructural parameters at the cellular and subcellular level. The Klumperman laboratory recently published the first example of CLEM from live cells to 3D EM at the level of a single organelle. The 3D EM data (e–g) are obtained by FIB-SEM of the ROI identified in live cells (a–c) and fluorescence microscopy (D). Reprinted by permission from: Fermie, et al. “Single organelle dynamics linked to 3D structure by correlative live-cell imaging and 3D electron microscopy.” *Traffic* 19.5; 2018): 354–369.

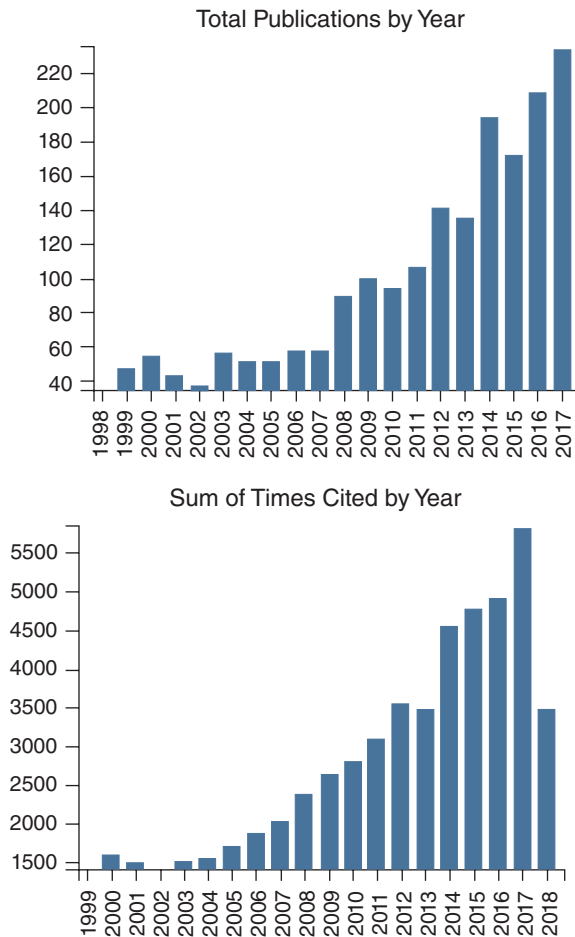


Figure 1.7 Citation report generated from Web of Science Core Collection between years 1900–2017 for 4,139 publications, which have the words *correlative*, *light*, *electron*, and *microscopy* either in their title or topic.

two-photon [106], fluorescence recovery after photobleaching (FRAP) [107], cryo-LM, and cryo-EM [108], live-cell imaging and immunogold labeling [109], fluorescence in situ hybridization (FISH) [110], cryo-scanning transmission electron microscopy (cryo-STEM) [111], and most recently super-resolution microscopy [112, 113, 114, 115, 116, 117, 118]. For a long time, CLEM had remained a 2D technique, at least at the EM end. With the advent of TEM tomography, this changed, and many correlative LM-TEM tomographic studies have opened up the third dimension at the nanoscale [119, 120, 121, 122, 123, 124, 125, 126]. More recently, other 3D EM techniques have been used for CLEM, such as: serial section TEM (ssTEM) [127, 128], array tomography [129, 130], focused ion beam SEM (FIB-SEM) [131, 132, 133, 134], and serial blockface imaging (SBFI) [135]. Fermie et al. demonstrated correlation of live-cell imaging to 3D EM, and linked dynamic characteristics of single organelles to their 3D ultrastructure [136](Figure 1.6). Other imaging techniques have also been used to enhance LM and EM studies, most often the use of CT or soft X-rays produced by either synchrotron beamlines or standalone instrumentation [137, 138, 139, 140].

CLEM came about because scientists needed more information than either LM or EM could offer on its own. The technique began slowly and it was some time before the

hardware and software available could fully support its development. What began as a few papers showcasing the significant efforts of some intrepid microscopists has now become a rapidly growing body of literature (Figure 1.7). It took many years for photons and electrons to learn to work together, now that they have, the future for correlative microscopy looks bright indeed.

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