Integration Without Compromise

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Introduction

Correlative microscopy is the combination of light and electron microscopy, commonly known as CLEM. The reason why the combination of these two modalities is of such interest is because they can provide a wealth of complementary information.

Fluorescence microscopy (FM) is well known for its diversity and ability to label almost any biologically relevant structure. One of the advantages is that one can simultaneously label different molecules with fluorophores that emit different colors. This makes it possible to study the functioning and interaction of many cellular processes.

Electron microscopy (EM) is the method of choice when one needs structural information at the nanometer scale. Because the wavelength of accelerated electrons is so much shorter than that of visible light, much smaller features can be resolved. But what distinguishes light from electron microscopy is not only the resolution; the type of contrast that one typically measures in EM is very distinct from FM. Whereas in FM one labels specific macromolecules, and only this macromolecule is detected, in EM one acquires primarily contextual information. Examples include membrane structures such as the endoplasmic reticulum, the Golgi apparatus, and vesicular structures.

CLEM. The potential of CLEM lies in the combination of these two modalities: multi-color labeling in combination with high-resolution contextual information. Traditionally CLEM is performed by combining the results from these two different microscopy modalities, acquired using separate instruments, at separate locations, using possibly different sample preparation protocols. That approach results in procedures that are notoriously time-consuming and require high levels of expertise. Furthermore, creating an accurate unbiased overlay requires an independent set of features,which can be used to align both modalities.

Integrated CLEM. With the introduction of integrated CLEM, that is, the combination of a light and electron microscope in a single machine, most of these difficulties can be overcome. With an integrated approach, switching between operating modalities is seamless and extremely easy. Furthermore, the alignment between EM and FM can now be achieved with high accuracy, completely automatically, and without any additional alignment fiduciary marks or landmarks.

Materials and Methods

SECOM platform. The SECOM platform is an integrated correlative light and electron microscope developed by DELMIC BV (Delft, the Netherlands). The SECOM combines a high-end optical fluorescence microscope with an existing scanning electron microscope (SEM) (Figures 1A and 1B). This platform originates from the charged particle optics group from Delft University of Technology in the Netherlands [1, 2]. After its initial technical development, the first biological measurements were conducted in collaboration with Erasmus University Medical Center [2] and Leiden University Medical Center [3]. The features of the SECOM platform are all closely related to its integrated design. From the start, the system is designed to be uncompromising for both light and electron imaging. It is possible to use high-NA light microscope objectives and almost any type of SEM detector. A unique feature is the alignment procedure of the light and electron images. Thanks to the integrated design, it is possible to automatically align both modalities to each other with an accuracy of better than 50 nm.

Image correlation and alignment. Correlating functional information of FM with structural information of EM is one of the main motivations for using CLEM.

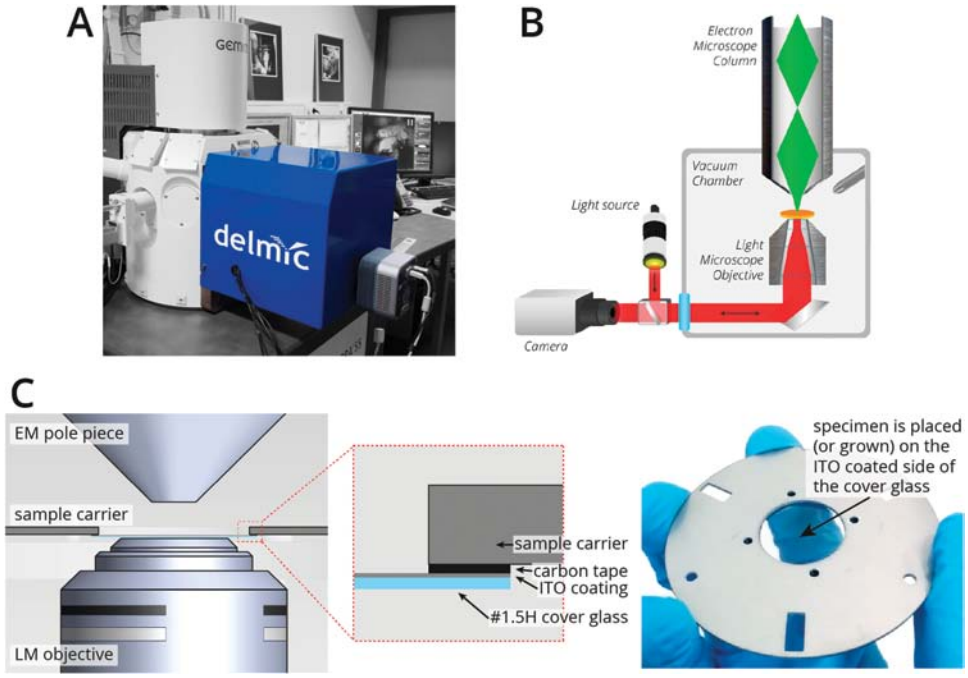
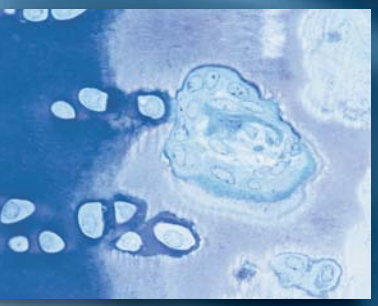


Figure 1: (A) SECOM platform installed on a Zeiss SEM. (B) Schematic representation of the electron optics (in green) and light path (in red). (C) Samples can be placed on the SECOM platform using ITO-coated cover glasses using a sample carrier ring.



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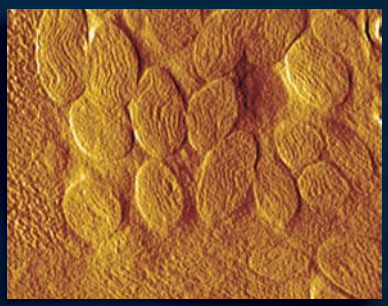

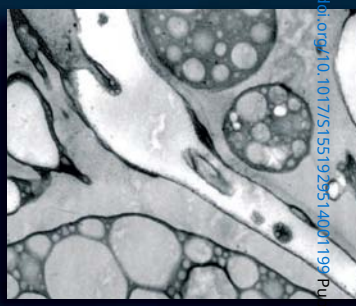

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In order to achieve this correlation, images from different modalities need to be aligned and overlaid. This alignment is far from trivial because the contrast that is generated can be very different. Sometimes it is possible to recognize the structure of interest both in EM and FM, but this is a challenging procedure because one takes the risk of accidentally introducing confirmatory bias, that is, a hypothesized correlation is used to confirm the hypothesis. To avoid this risk, one needs to use an independent alignment procedure. Possibilities include: mechanical alignment, which is limited to a repeatable accuracy of ~10 µm; using extra labeling such as DAPI (4',6-diamidino-2-phenylindole) or additional correlative fiducials, which adds to the already difficult sample preparation; or using cathodoluminescence (CL), the procedure used by the SECOM platform.

Integrated specimen preparation. Another difficulty is the problem introduced by intermediate sample preparation steps. In non-integrated CLEM, it is quite common to use intermediate EM staining when switching from FM to EM. This step can severely alter the conformation of the sample, for example, by local non-uniform shrinking. These local deformations severely hamper reliable correlation of subcellular structures. These problems can be avoided by using a sample preparation protocol without an intermediate staining step when switching to EM. This does require that this integrated sample preparation produces contrast for EM and FM simultaneously. For integrated CLEM, integrated sample preparation is essential, but because of the increased overlay accuracy we strongly advocate the use of integrated sample preparation even when used in a non-integrated setup.

Cathodoluminescence for alignment. Alignment of the EM and FM images in the SECOM platform is performed using CL. When electrons impinge on a luminescent material (such as glass), photons are generated (illustrated in Figure 2A). In the SECOM, this CL light can be detected using the microscope objective, which is situated directly under the sample and pole piece of the SEM. When the electron beam of the SEM is positioned at a predefined location, a spot of CL light is detected with the light microscope. In this way, the location of the electron beam can be exactly correlated to a position in the light image.

A single CL spot at the optical axis of the SEM can be used to align the optical axes of the light and electron paths. The SECOM performs this alignment by moving the light objective using a pair of piezo-actuators. After the optical axis is aligned, the alignment procedure using the CL spots is extended to position a grid of non-permanent CL markers in the field-of-view shared by the light and electron microscope. Figure 2B shows an example of such a grid of CL spots measured with the light microscope. For an accurate alignment, the location of these spots needs to be known accurately in both the light and electron image. For the electron image, the position of the spots is well known because they are positioned using the same mechanism that is used to scan the SEM image. In the light image, the location of the spots is measured by automatically fitting the location of these spots in the image using basic image processing procedures. Using the corresponding positions of these spots in a grid, in both the light and electron image the alignment procedure can correct for translation, scaling, rotation, and possibly non-linear distortions. The alignment of the light image with respect to the electron image is stored as metadata in the saved images and used to display the overlay correctly in the graphical user interface. The accuracy of this alignment has been quantified to be better than 50 nm [4].

Results

Application example 1: bird brain sections. Neurology is an interesting example for correlative microscopy because it is an ideal tool for studying the connectivity of neurons at a resolution sufficient to identify synaptic connectivity. Using fluorescent tracers, specific types of neurons can be followed over large distances. Correlative microscopy offers the possibility of identifying individual synapses with neurons originating from a different part in the brain [5].

Figure 3A shows sections of songbird brain, measured with the SECOM platform. Samples were kindly provided by T. Templier and R.H.R. Hahnloser. Details about sample preparation can be found in a publication by D Oberti et al. [5]. In short, fluorescent tracers (Dextran Alexa 488 and Dextran Texas Red) were injected at specific locations in the brain of the live songbird. After a survival time of 5 days, a lethal dose was injected and perfused through the heart, followed by fixation using a combination of paraformaldehyde and glutaraldehyde. The brain was then removed and post-fixed, after which 60 µm thick sagittal sections were cut. Using widefield fluorescence microscopy, the area of interest was localized. The sections were then post-fixed in potassium ferrocyanide, osmium tetroxide, and uranyl acetate. After dehydration, the sections were

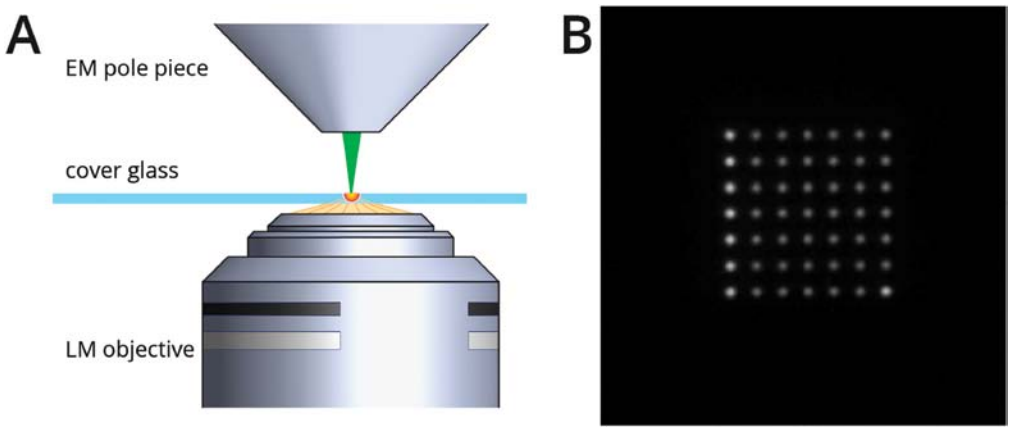

Figure 2: (A) Schematic depiction of cathodoluminescence. The e-beam (in green) impinges on the glass, which produces photons that can be imaged using the LM objective. (B) By writing a grid of CL spots, the automatic alignment procedure can correct for translation, scaling, and rotation between LM and EM.

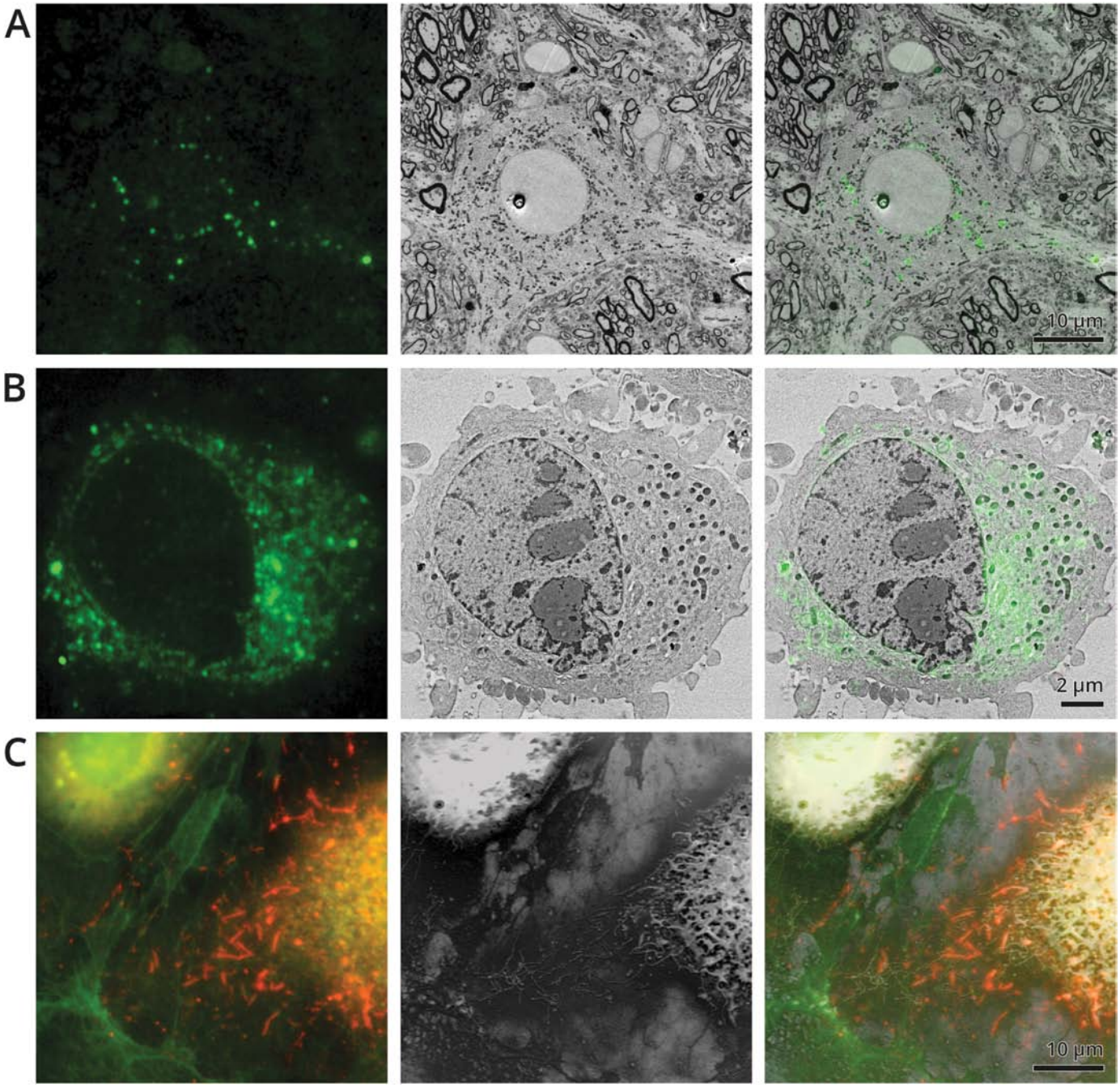

Figure 3: CLEMs using the SECOM platform (DELMIC B.V.) installed on a Quanta 250 FEG (FEI Company). First column: fluorescence images. Second column: scanning electron micrographs. Third column: overlays of FM and EM. (A) Projection neurons in songbird brain (tracers: Dextran Alexa 488 and Dextran Texas Red, immuno-labeling: rabbit anti-Alexa 488 and rabbit anti-Texas Red primaries, goat anti-rabbit secondary conjugated with Alexa 546). (B) HeLa cell expressing GFP-C1. (C) Human umbilical vein endothelial cells labeled for VWF and filamentous actin (rabbit anti-human VWF primary, goat anti-rabbit secondary conjugated with Alexa 568; Phalloidin-Alexa 488). Technical details (A) and (C): EM imaging using secondary electron detector and FM imaging with Nikon Plan Apo 60×/0.95 lens, multicolor LED light engine, and Clara CCD camera (Andor Technology). B) EM imaging using the vCD backscatter detector and FM imaging with Nikon Plan Apo 100×/1.40 oil immersion lens using vacuum-compatible immersion oil, laser light source, and Zyla sCMOS camera (Andor Technology).

embedded in Durcupan ACM resin (Fluka, Buchs, Switzerland). After curing, 60–90 nm thick serial sections were cut and put onto indium tin oxide (ITO) coated cover glasses. For immunofluorescence, the sections were first etched with periodic acid and then relabeled to identify the injected tracers (rabbit anti-Alexa 488 and rabbit anti-Texas Red primary antibodies and goat anti-rabbit secondary antibody conjugated with Alexa 546). The cover glasses were then mounted in special carrier rings designed for the SECOM platform, shown in figure 1C. Data was then collected on the SECOM platform, installed on a Quanta 250 FEG SEM (FEI Company), using a Nikon Plan Apo 60× /0.95 objective and a multicolor LED light engine. SEM images were collected using the secondary electron detector, and fluorescence images were captured using a Clara CCD camera (Andor Technology).

The results show that the EM staining results in good observable contrast. The fluorescence labeling is also easily

detectable, but its intensity did decrease to unusable levels in a few days. This example is a good illustration of a protocol for neurology samples. Nevertheless, with some alterations, it might prove to be useful in other fields as well.

Application example 2: HeLa cells expressing GFP. A recent study by C J Peddie et al. [6] provides another good example of sample preparation for integrated correlative microscopy. The interesting aspect of this protocol is that GFP and mCherry fluorescence is preserved after embedding. This opens up many opportunities for biological systems that rely on genetically encoded fluorophores.

Figure 3B shows HeLa cells expressing GFP-C1, imaged using the SECOM platform. Sample preparation and data acquisition were performed by C J Peddie and L M Collinson. The authors note that one of the key elements in preserving GFP fluorescence might be the use of a quick freeze substitution protocol, adapted from [7]. Sample preparation details are described in [6]. In short, transfected cells were fixed using a high-pressure freezer. The samples were then freeze-substituted using a quick freeze-substitution protocol. After the substitution, the samples were embedded in HM20 and polymerized. Serial sections of 200 nm were cut and collected on ITO coated coverslips. Images were acquired on a SECOM installed on a Quanta FEG 250 (FEI Company) using the vCD backscatter detector, a Nikon Plan Apo 100× /1.4 objective using special vacuum-compatible immersion oil (developed by DELMIC) and imaged using a Zyla sCMOS camera (Andor Technology).

Application example 3: HUVECs on indium tin oxide (ITO). The last application example shows how correlative microscopy on whole cells provides a fast and straightforward method to study cell morphology. In a recent study, Liv et al. [2] showed that a very simple protocol for whole cells can be used to accomplish FM and EM contrast simultaneously. For example, this protocol was used in a study in which correlative measurements of whole cells cultured on a substrate could quickly provide answers about the functional and morphological characteristics of different cells [3].

Here we tested this protocol on human umbilical vein endothelial cells (HUVEC). Samples were stained for actin and Von Willebrand Factor (VWF). Figure 3C shows that organelles storing VWF (Weibel-Palade bodies) can be easily identified thanks to the fluorescence. It is worth noting that the fluorescence of this sample is very well preserved. Even after storing the dried sample in a refrigerator for half a year, the sample still contains sufficient amounts of fluorescence.

The cells were seeded directly onto ITO slides and grown to desired confluency. The cells were then fixed using a combination of paraformaldehyde and glutaraldehyde. Permeabilization was performed using Triton X-100 and blocking with normal goat serum. Cells were immunolabeled for VWF using rabbit anti-human VWF primary antibody and goat anti-rabbit secondary antibody conjugated with Alexa 568, and stained for filamentous actin with Phalloidin-Alexa 488. The cells were then dehydrated in a graded ethanol series and finally air-dried. Correlative measurements were collected using the experimental setup described in the application example 1.

Conclusion

We have shown that integrated sample preparation for FM and EM facilitates accurate and reliable subcellular correlation. We have demonstrated that it is possible to prepare samples that show both FM and EM contrast simultaneously. With the use of non-permanent CL markers, it is no longer necessary to introduce an additional set of correlative alignment markers. Furthermore, because alignment using CL works on any sample and is fully automated, it ensures that there is no longer a possibility to accidentally introduce a confirmatory bias.

Acknowledgments

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References

[1] AC Zonnevylle et al., *J Microsc* 252 (2013) 58–70.
[2] N Liv et al. *PloS one* 8 (2013) e55707.
[3] PW Voorneveld et al., *Gastroenterology* 147 (2014) 196–208.
[4] MT Haring et al., article in preparation.
[5] D Oberti et al., *Frontiers in Neuroanatomy* 4 (2010) 24.
[6] CJ Peddie et al., *Ultramicroscopy* 143 (2014) 3–14.
[7] KL McDonald and RI Webb, *J Microsc* 243 (2011) 227–33.

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