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Integrated Microscopy: Highly Accurate Light-Electron Image Correlation Anywhere on a Sample

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Superresolution fluorescence has pushed the resolution of light microscopy (LM) towards length scales traditionally accessible only with electron microscopy (EM)[1]. Also, developments in scanning EM (SEM) have moved image dimensions for EM to typical LM fields-of-view[2] and into the third dimension[3]. By correlating data from both techniques[4], molecules can be localized within the context of cells and tissue and with reference to their live dynamics, but throughput and quantification are hindered by elaborate, expert procedures involving separate microscopes. We have developed an integrated approach with high-numerical aperture LM inside an SEM, such that the electron beam can be positioned anywhere within the fluorescence field of view[5, 6]. Here, we will show that this approach allows for automated light-electron overlay, i.e. without fiducials or user data interpretation, with the same high accuracy anywhere on the sample[7].

Our integrated microscope consists of an inverted fluorescence microscope with sample translation stage replacing the original sample stage in an SEM. Using vacuum compatible immersion oil, numerical aperture of the LM can be up to 1.4. The axes of both microscopes can be aligned to about 1 μm[5]. Samples containing fluorescence can be prepared either via standard chemical fixation procedures followed by post-embedding immuno-labelling, or using an adapted sample preparation protocol for maintaining genetic expressed fluorescence during EM sample preparation[4]. In all cases, integrated microscopy allows for rapid identification of regions of interest for SEM based on fluorescence expression and seamless exchange between both fluorescence and electron microscopy acquisition[6]. However, both modalities are still separated imaging systems, each with their own field distortions. Accurate correlation needs a registration procedure to determine both the relative position and orientation of both image fields, as well as to correct for microscope distortions.

Image acquisition in the integrated microscope is schematically indicated in Fig. 1. For automated overlay, we exploit the fact that the focused electron beam generates (cathodo-)luminescence in the ITO-coated glass substrate that supports the sample. When the electron beam is scanned over several well-separated positions, this leads to an array of circular intensity spots, or pointers, on the LM camera (Fig. 1-III). We obtain a discrete set of LM coordinates by fitting pointer centre positions for each pointer. These LM pointer coordinates can be linked to the a priori set electron beam positions, and thus the full LM-SEM coordinate transformation can be determined. To achieve a high overlay accuracy, the distortions between LM and SEM imaging systems need to be mapped. This we achieve by repeating this procedure for a large number of pointers and extracting the non-linear contribution to the coordinate transformation (Fig.1 right panel). Moreover, for each region of interest, the LM-SEM overlay accuracy now depends on the number of pointers used, which as we will show, can go down to 5 nm, i.e. bio-molecular length scales. As this procedure can be conducted anywhere on the sample and in a fully automated fashion, this paves the way towards large-scale highly accurate image correlation [8].

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[8] Our integrated microscope served as a prototype for a commercial system by Delmic BV. A. C. Zonnevylle, P. Kruit, and J. P. Hoogenboom are shareholders in Delmic BV.

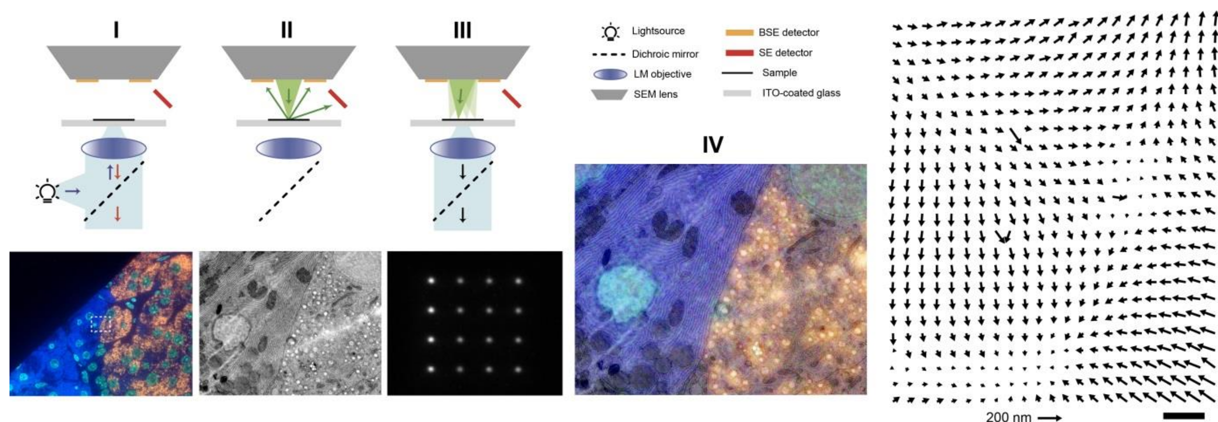

Figure 1. Image acquisition and image registration in an integrated microscope. First, (I) large field-of-view LM image is collected. Based on this image, one or multiple regions of interest (dashed rectangle) for (II) SEM are determined. After collection of LM and SEM images, (III) the electron beam is positioned on specific positions in the EM field of view and electron-excited luminescence is recorded. By centre fitting the LM intensities, LM coordinates can be mapped to the pre-set SEM coordinates. In this way (IV) an overlay image can be created. Right: Example of the distortions between FM and SEM coordinate systems, measured over a large (LM) field-of-view using this overlay procedure. Tissue sample courtesy of P. de Boer, B. Giepmans (UMC Groningen).

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