Find Your Way with X-Ray: microCT Guided Correlative Microscopy

M.A. Karreman[1,2,3], Markus Heneka[4], Jens Waldeck[5] and Y. Schwab[1]

1 European Molecular Biology Laboratory, Cell Biology and Biophysics Unit, Heidelberg, Germany
2 Department of Neurology, University Hospital Heidelberg, Heidelberg, Germany
3 German Cancer Research Center (DKFZ), Heidelberg, Germany
4 RJL Micro & Analytic GmbH, Im Entenfang 11, 76689 Karlsdorf-Neuthard, Germany
5 Bruker BioSpin MRI GmbH, Rudolf-Plank-Str 23, 76275 Ettlingen, Germany

In life science, developmental biology, and medicine there is an ever-increasing need to visualize biological events in their native context at a high resolution [1-4]. Intravital correlative microscopy (IVM) enables users to follow biological processes in vivo using fluorescence microscopy (FM) and subsequently reveal the object of interest and its microenvironment at high resolution with electron microscopy (EM).

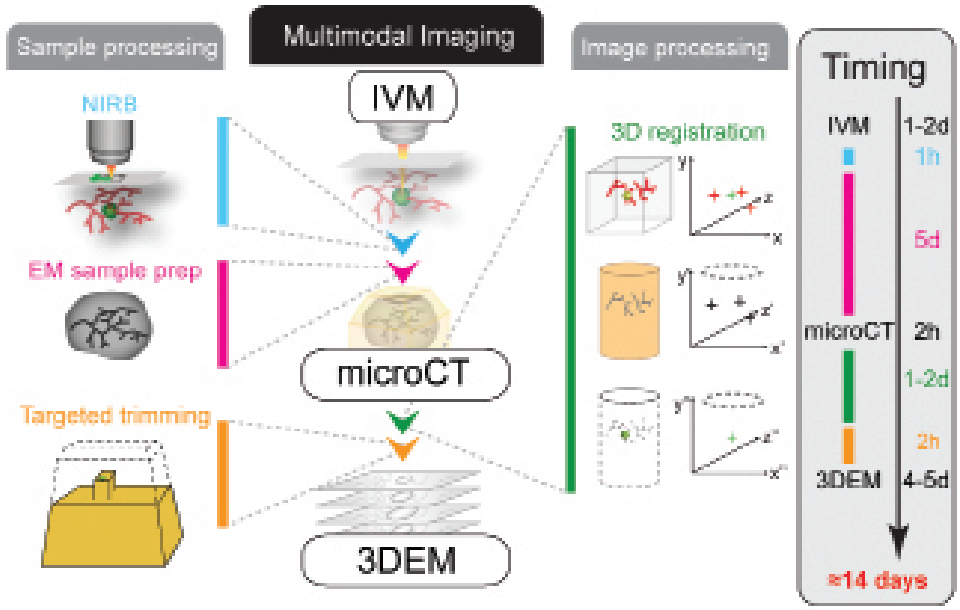
The main challenge for correlative microscopy is keeping track of the volume of interest while moving from one imaging modality to the next. Typically, difference in image formation and field of view (FOV) between FM and EM make it difficult to correlate datasets. In addition, conventional sample preparation procedures for EM cause loss of fluorescent labels and generate changes to the orientation and dimensions of the sample, often making it impossible to correlate datasets afterwards. For these reasons, the retrieval of the ROI is the most difficult, tedious and time-consuming step of correlative microscopy.

In this technical note, we describe a robust and rapid workflow that relies on microCT to map electron-dense, resin-embedded biological EM-samples [5-7]. In brief, following (intravital) fluorescence microscopy (FM), the sample is processed for EM. Subsequently, microCT scans are obtained and correlated to the FM volumes. 3D registration of both datasets enables users to accurately approach this area using ultramicrotomy. Primarily, the protocol enables one to correlate 3D FM to EM and is applicable to a wide variety of biological samples. It enables employing endogenous landmarks for correlation, and therefore does not require the introduction of artificial markers that potentially disrupt the sample. Secondly, microCT can also be used to reveal the orientation of electron dense, opaque samples inside the resin block. This microCT map guides the alignment of the sample in the ultramicrotome, and allows users to accurately trim the sample for subsequent thin-sectioning and/or volume Scanning EM [8,9].

Materials & Methods

Dynamic processes in cells and organisms can be monitored over time with fluorescence microscopy (FM) in vivo. In this technical note, we exemplify the procedure with

Figure 1



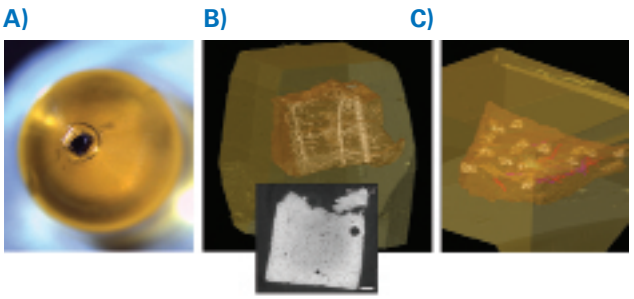
Sample preparation, multimodal imaging and image processing workflow. By use of an optimized workflow concept the processing time could be reduced from 3-6 months to approximately 2 weeks, while gaining highly relevant data at the same time. IVM: Intravital Microscopy; 3DEM: 3D electron microscopy. Reproduced from Karreman et al, 2016 [5].

our study of the early steps in the brain colonization of metastatic cancer cells in mice [5,6] (Figure 1). These events are visualized in vivo by multi-photon microscopy through a chronic cranial window [10]. Upon identification of the event of interest, the mouse is perfusion-fixed and the ROI is subsequently marked on the surface of the brain via near-infrared branding (NIRB)[11].

The NIRB markings are visible on the fixed brain with a stereomicroscope, which allows one to dissect a ~1-2 mm3 biopsy containing the volume of interest. The biopsy should be cut asymmetrically, to be able to recognize the orientation of the sample following EM processing. In doing so, during the final embedding step of the EM processing protocol the sample can be mounted with the branded side closest to the resin block surface (Figure 2). The EM processing protocol entails fixation and infiltration with 1% osmium tetroxide (OsO4) and 1.5% potassium ferrocyanide, followed another fixation by 1% OsO4, and staining with 1% aqueous uranyl acetate. These heavy metals stabilize the sample and generate a strong image contrast in EM and microCT. Using lower concentrations of stains will still allow one to see the details in microCT that are required to map the sample. Depending on the choice of stain and the type of sample, different tissue components can be visualized with microCT [12].

The biopsy is then dehydrated in a graded series of ethanol in water and gradually infiltrated with epoxy resin. To improve infiltration efficiency and generally speed up the procedure, these steps were performed in a laboratory-grade microwave. Following polymerization of the epoxy resin, the

Figure 2



Mouse Brain and Skin. A: Resin block with embedded mouse brain tissue harboring the ROI. Note the tissue is fully opaque due to the 3DEM processing protocols. B: Reconstructed and rendered microCT volume of the sample

sample is trimmed around the biopsy to generate a small sample in the range of 2 x 2 x 4 mm, with a flat blockface and straight sides (see Figure 1).

To reveal the orientation and architecture of the biopsy inside the resin block, the sample is imaged with the Bruker Skyscan 1272 microCT (Bruker BioSpin MRI GmbH, Ettlingen, Germany). The sample is then mounted on a standard holder, aligned and positioned close to the X-ray source to achieve highest-possible resolution. Typically, the sample is scanned with 50 kV / 200 µAmp or 75 kV / 133 µAmp, without filter, at scanning angles in the range of 180o, with

an 1-2 µm isotropic voxel size. The imaged volume is then reconstructed using NRECON software (Bruker BioSpin MRI GmbH, Ettlingen, Germany). Importantly, microCT imaging of the EM-processed sample reveals structural details that can subsequently be exploited for correlation with the FM volume. MicroCT reveals the cell nuclei, the outlines of the biopsy and the resin block and, importantly, the vasculature of the mouse brain. Since the blood vessels are also visualized during in vivo imaging, these can then be used as endogenous landmarks to perform the correlation and 3D registration. Importantly, different cells and tissue types reveal different features in microCT [5], and in some cases the region of interest (ROI) itself can already be distinguished [8].

In 3D image processing software, such as Amira, 3D models of the imaged volumes are generated by semi-automatic segmentation of the datasets. Using the landmark module equal positions in both datasets are selected, which allows the user to register the IVM model into the microCT model using landmark surface warp module. The position of the ROI, from the IVM volume, is now mapped with respect to the outlines of the EM-processed resin block shown in the microCT volume. Using digital measuring tools the distance in X, Y and Z from the surfaces of the sample block to the ROI, a tumor cell can now be determined. The small resin block is mounted on an empty slab of resin and can be trimmed in an ultramicrotome to accurately approach the ROI. Upon reaching the position of the tumor cell, the sample is ready for subsequent serial sectioning or can be prepared for volume SEM (Figure 1).

Results & Discussion

Imaging a resin-embedded, electron dense biological sample with microCT allows the user to map its orientation within the resin block and importantly, reveals landmarks that can be exploited for correlative microscopy. We have successfully employed the workflow (Figure 1) on a variety of specimen, including various mice tissues, zebrafish, platynereis, and mice- and starfish oocytes.

The a-priori knowledge on the sample's architecture, provided by the microCT scan, allows efficient and accurate approaching of the region of interest (ROI) using microtomy. Without microCT guidance, a trained electron microscopist would need at least 3-6 months to retrieve a single tumor cell in a 1-2 mm^3-sized brain biopsy as described here, if correlation is even achievable. MicroCT thus speeds up correlative microscopy and 3DEM workflows, so that a higher number of events can be analyzed and significant conclusions can be drawn on rare but critical developmental or pathological events.

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Bruker BioSpin
info@bruker.com