Microtome-integrated microscope system for high sensitivity tracking of in-resin fluorescence in blocks and ultrathin sections for correlative microscopy

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Many areas of biological research demand the combined use of different imaging modalities to cover a wide range of magnifications and measurements or to place fluorescent patterns into an ultrastructural context. A technically difficult problem is the efficient specimen transfer between different imaging modalities without losing the coordinates of the regions-of-interest (ROI). Here, we report a new and highly sensitive integrated system that combines a custom designed microscope with an ultramicrotome for in-resin-fluorescence detection in blocks, ribbons and sections on EM-grids. Although operating with long-distance lenses, this system achieves a very high light sensitivity. Our instrumental set-up and operating workflow are designed to investigate rare events in large tissue volumes. Applications range from studies of individual immune, stem and cancer cells to the investigation of non-uniform subcellular processes. As a use case, we present the ultrastructure of a single membrane repair patch on a muscle fiber in intact muscle in a whole animal context.

A key challenge in biomedicine and biotechnology is to link the function of genes from their phenotypic effects in an organ and tissue context to the molecular events occurring at the subcellular level. Imaging is a central technology to understand these processes in space and time. The coverage of the necessary wide ranges of spatial and temporal resolution requires the combination of different imaging modalities. Fluorescence microscopy allows imaging of biological specimens with high resolution offering also measurement of real-time kinetic data in living systems. A disadvantage of fluorescence microscopy is that the investigator remains blind to the non-fluorescent cellular context including elements like filaments, vesicles, membranes, and organelles1,2. For high resolution imaging of these cellular structures, electron microscopy and tomography are the imaging modalities of choice. In other instances, two light based modalities such as, for example, confocal microscopy and super resolution fluorescence microscopies have to be combined3–7. In many instances, switching from one modality to another entails the processing of the specimen such as fixation, embedding in resin, sectioning, staining etc. A particular critical issue is the tracking of the ROI without losing its coordinates during the processing and transfer. When rare or unique events in intact tissue or organisms are to be investigated, an additional complication is presented by the large tissue volume in which the ROI has to be found.

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In recent years, huge progress has been made to tackle the challenge of correlating light and electron microscopy (CLEM) imaging. However, these protocols are anything but routine yet. Issues are specimen fixation and processing for electron microscopy and their effects on the preservation of fluorescent signal or cellular detail. The challenge posed by "rare" events or structures that are represented by single or a few cells only in a large piece of tissue calls for further developments. Examples are metastasizing cancer cells, stem cells, macrophages in their natural tissue context or a single myofiber with a membrane lesion in an intact muscle. Due to the narrow field of view of high-resolution imaging methods, a prerequisite to find such "rare" structures is the trimming of the tissue block down to the ROI accompanied by efficient tracking methods in the subsequent processing of the sections cut from the tissue block.

Here, we report the construction of a new instrument that overcomes many of the above-mentioned technical hurdles and greatly facilitates the tracking and capture of scarce structures/events. A compact and highly sensitive epifluorescence microscope was developed and mounted onto an ultramicrotome to perform both fluorescence tracking and imaging on a single instrumental setup and at each step of the specimen preparation protocol. While another set-up was reported recently, our Microtome-Integrated-Microscope (MIM) is characterized by a remarkable sensitivity of in-resin detection of fluorescence by employing a new design of a light weight microscope optimized for photon collection. By having integrated this microscope directly onto the ultramicrotome, one can follow the ROI at each step from specimen localization in the block, block trimming, sectioning, tracking sections with fluorescence to documentation of landmarks and relative coordinates of the ROI. We have developed methods to assess the precise 3D location of the ROI in the block reducing sectioning efforts enormously. The MIM is highly suitable for detecting weak fluorescence signal or localizing rare events in large tissue pieces or small organisms.

We applied our new method to study the ultrastructure of the membrane repair patch after muscle membrane wounding in the intact muscle. Repair proteins and lipids rapidly accumulate at the site of wounding forming a tight patch re-establishing the integrity of the barrier to the extracellular space. In skeletal muscles of zebrafish embryos, this repair patch is removed by macrophages as a prerequisite for the complete restoration of the lipid bilayer of the sarcolemma. The damaged plasma membrane has previously been subjected to ultrastructural analysis in tissue culture cells and sea urchin eggs. However, none of the previous studies focused on the repair of an individual damaged cell in situ in the tissue context of a healthy animal at the ultrastructural level. We show that the repair patch in vivo consists of an amorphous mass and displays various vesicle profiles and membrane stretches. Moreover, for the first time, we show here ultrastructural details of a macrophage interacting with a membrane repair patch. These user cases demonstrate that the MIM is optimally suited to reliably detect single cellular and subcellular structures in large tissue volumes or in intact zebrafish embryos.

**Results**

**Design features of the microtome-integrated microscope (MIM).** It is critical that fluorescence signals can be detected in the 3D space of the tissue block before and during sectioning. To this end, the MIM was mounted on the head of an ultramicrotome (Leica, UC7) perpendicular to a stereomicroscope (Fig. 1a–c). This configuration allows switching from trimming and cutting under the stereomicroscope to image acquisition with the MIM by simply rotating the microtome head.

The MIM was designed to image the sample through air with long working distance objectives to avoid physical contact with the fragile sections. To detect faint fluorescence signals even deep in the tissue block, we optimized the MIM for light sensitivity: (1) A short phototube was built with a minimum of lenses and (2) connected to an ultra-sensitive camera (sCMOS) (Fig. 1a–c). The optical tube was equipped with a plan apochromatic lens (diameter, 22 mm; focus 200 mm) to be used with standard infinity corrected objective lenses. Moreover, the tube lens was corrected for three colors facilitating the detection of overlapping fluorescent signals of different colors.

The MIM has a low weight and is thus easily moved via a 3-axis stage over a large area to examine the resin block, the sections floating in the boat or the sections transferred onto EM grids. The microscope's position in Z is set by a motorized and computer-controlled focussing actuator (Newport SMC-100CC) with micrometer block, the sections floating in the boat or the sections transferred onto EM grids. The microscope's position in lens was corrected for three colors facilitating the detection of overlapping fluorescent signals of different colors.

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a micromanipulator (Supplementary Fig. 2a). The MIM was focused on the grid surface illuminated from above with reflected white light (Supplementary Fig. 2b). The grid was removed and the MIM was then focused on the fluorescent ROI (Supplementary Fig. 2c). The difference between these two positions was then calculated by taking into account both grid thickness and the refractive index of Lowicryl HM20 to compensate for the axial focus.
shift (Supplementary Fig. 2d–f). We measured positions of ROIs at least 200μm deep in the resin block with an accuracy of 1 to 2μm. By employing the DEAP method, we reduce sectioning and subsequent scanning of ribbons and sections significantly.

The optical properties of the MIM. As assessed with a glass lamella micrometer, the magnification of the optical tube (M_{tube} = 10) of the MIM and its camera is close to that of a standard epifluorescence microscope. The total magnification (M_{total}) is given by the product of the magnification of the objective (M_{obj}) and the optical tube (M_{tube} = 10). The overall magnification ranges from 50- to 1000-fold. The MIM provides working distances between 3.2 and 12 mm and a field of view between 2.6 mm × 2.6 mm to 135μm × 135μm with a pixel size from 1.28 to 0.063μm (Table 1).

We first assessed the performance of the MIM by comparing it with a standard confocal microscope (Leica SP5). We chose a confocal microscope as reference since confocal imaging is the method to detect fluorescence signals deep in the tissue of our application model, the intact zebrafish embryo. We cut 90 and 350 nm thick parasaggital sections through a 3-day old zebrafish embryo expressing membrane-tagged mCherry (Tg(kdr:Hsa.HRAS-mCherry)) in blood vessels. For comparison, we imaged the adjacent sections from the same transgenic embryo under a confocal microscope. The MIM yielded brighter signals from sections of the same thickness than the confocal microscope (Fig. 2a–d). When using the vessel walls as reference, the lateral resolution of the MIM is comparable to that acquired in the confocal system with 80 nm pixel size (Fig. 2e–f; for details of the settings of the confocal microscope, see legend of Fig. 2). Yet, the confocal system under the given settings requires approximately four times thicker sections to match the same signal-to-noise levels of the MIM.

To further assess the ability of the MIM to image weak fluorescent signals, we measured the light transmission of the MIM phototube (85%) is higher than that of standard epifluorescence wide field microscopes. We replaced the cameras by a light intensity sensor and used the same light source and objective on each of the three tested instruments (Fig. 2m). The light transmission of the MIM phototube (85%) is higher than that of standard epifluorescence microscopes (67% and 61% for Leica DMR and Leica DML, respectively) (Fig. 2n).

We calculated the theoretical lateral resolutions of the different lenses used on the MIM (Table 1). In addition, we measured the lateral resolution for the 40x air objective (NA 0.6) using 100 nm fluorescent beads and 610 nm excitation light. Based on a 2D PSF, the lateral resolution of the MIM is 97.8 nm under these conditions. The resolution across the field of view and vignetting effects are dependent on the objective rather than the phototube itself. Since the MIM employs commercially available plan apochromate objectives (Table 1) vignetting effects should be marginal. In agreement, we did not observe vignetting effects with a apochromate lens and also the field of view was homogenous (Fig. S5). In summary, the improved light transmission properties together with the integrated CMOS camera system make the MIM a highly sensitive device to detect fluorescent signals in resin-embedded tissues and cells.

Multi-step MIM workflow for CLEM. The MIM allowed us to develop an innovative CLEM strategy from cryo-fixation to electron microscopy (Fig. 3a). This concise workflow referred to as “Direct-CLEM” considerably reduces sample handling as compared with other CLEM protocols. Moreover, it offers high flexibility in connection with other CLEM strategies such as grid scanning with immersion objectives, block-face imaging in a SEM, TEM, electron tomography or super-resolution imaging.

As an example, we chose the ultrastructural analysis of a membrane repair patch present on a single myofiber in intact zebrafish muscle. Individual myofibers were tagged with fluorescent membrane repair proteins AnxA2-mEosFP (green) and Dysf-mCherry (red) and a membrane lesion was inflicted by pulses with an infrared laser as previously described. The trunk/tail regions of the embryos with the injured myofibers were cryo-immobilized by high pressure freezing. By choosing vitrification as method of tissue fixation, we reached a close-to-native ultrastructure and excellent preservation of fluorescence. The fluorescence of both mCherry and mEosFP were well maintained in the Direct-CLEM workflow.

After “clean-cutting” the surface of the resin block, the depth of the ROI in the block was determined with micrometer precision using the DEAP method. Predetermined the depth of the ROI with the DEAP method allowed rapid trimming of the block down to the depth of the ROI (Fig. 3b) followed by careful sectioning and collection of serial sections from the selected volume containing the ROI. For electron tomography, 250–260 nm thick sections were cut and ribbons of serial sections floating in the diamond boat were directly scanned for the presence of the repair patch using the 10x and 40x objectives (Fig. 3c). After transfer of positive sections onto formvar-coated EM-slot grids, the identity of the cell with the repair patch was unequivocally confirmed by the detection of both red (Dysf-mCherry, entire myofiber) and green (AnxA2a-mEosFP, repair patch) (Fig. 3d–f). Images of the fluorescent repair patch and of three reference points were then acquired with the 20x objective to record coordinates for detection of the ROI in the electron microscope (Supplementary Fig. 3c). Tilt series were acquired and tomograms of the repair patch at the wounded site were calculated (Fig. 3g,h, Fig. 4, Supplementary Movie 1). Taken together, this workflow illustrates the strength of the MIM which allows carrying out all necessary preparative and imaging steps with one instrument hence minimizing the risk to damage the fragile sections.

Ultrastructure of the membrane repair patch in a single muscle fiber of an intact zebrafish embryo. As a test case of the MIM and its associated workflow, we determined the ultrastructure of membrane repair patches in otherwise intact muscle tissue. Membrane lesions were introduced into individual myofibers of the somitic muscle of zebrafish embryos, and the repair patches were fluorescently tagged as described in the previous section. The Direct-CLEM workflow (Fig. 3a) was applied and the ultrastructure of six repair patches was reconstructed by electron tomography. The lesion patch from different injured cells did not have a uniform structure (Fig. 4a,b,d). From patch to patch varying membranous vesicular (Fig. 4c,g) and lamellar structures (Fig. 4e,f) of different size were imaged in an amorphous mass (Fig. 4b,d). The stacked membrane
structures observed are reminiscent of multilamellar bodies (Fig. 4e,f; Supplementary Movie 1, Supplementary Fig. 4). We previously showed that macrophages interact preferably with the repair patch of the damaged cells19.
The plasma membrane of the macrophage sitting next to the damaged cell (Fig. 4h) is tightly apposed to the repair patch forming a very tight interaction (Fig. 4i). Large vesicles reside close to the plasma membrane of the macrophage (Fig. 4h,i).

Taken together, these examples provide new insights into plasma membrane repair in an intact tissue context that have not been visualized at an ultrastructural level before and would not have been possible without the MIM. The MIM provides an unprecedented way to reliably find and image such “rare” and discrete structures embedded in a large tissue volume.

Discussion

Many areas of biological research demand the combined use of different imaging modalities for example to cover a wide range of magnifications or to record complementary information such as placing fluorescent patterns into...
Figure 4. High resolution imaging of the lesion patch by electron tomography. (a) Overview of muscle tissue with a membrane repair patch between two muscle fibers (arrow). Scale bar, 10 µm. (b) Tomographic slice through the repair patch in panel (a) at 9600x magnification. The sarcolemma of the lesioned cell (asterisk) is already resealed (black arrowheads). Note that, the sarcomere organization appears still disturbed. A characteristic, rather thick and slightly electron dense coat, is lining the sarcolemma extracellularly over the lesioned region (double black arrow) while the lower right myofiber seems also affected by the laser. Scale bar, 500 nm. (c) The lesion site shows heterogeneous vesicle profiles with a grainy or a smooth aspect (black arrow in c). Scale bar, 200 nm. (d) Tomographic slice of a large repair patch at 9600x magnification with heterogeneous membranous profiles shown in detail in panels (e,f,g). Membranes appear loosely stacked in a multilamellar configuration (black arrowheads in e,f). Scale bar, d, 500 nm; (e), 200 nm; (f), 100 nm; (g), 100 nm. (g) Some vesicles of various sizes are also visible forming a row against the lesioned myofiber. These vesicles also show heterogeneity in their aspect (black arrows in g) as noticed in (c). (h) Image of a macrophage sitting on the repair patch. The plasma membrane of the macrophage shows a close apposition with the repair patch (black arrow in h). Scale bar, h, 10 µm; (i), 1 µm. The damaged cell shows a stronger contrast in comparison to the surrounding undamaged cells and appears shrunk. This presumably reflects that the sarcolemma damage triggered an apoptotic program in the myofiber as previously described for cells with large lesions (Middel et al., 2016).

A high resolution ultrastructural context. A technically difficult and largely unsolved problem in CLEM is the transfer of the same specimen between different imaging modalities without losing the ROI14,25,27. Here, we report a new and highly sensitive microtome-integrated imaging system that combines a purpose-designed microscope...
The repair patch accumulates phosphatidylserine in a dysferlin-dependent manner. The latter serves as a lemma. Thus, the membrane of damaged myofibers seems to be repaired on the cell-proximal side of the patch. This tight association of the macrophage may help maintaining the integrity of the barrier function while excluding the repair patch in smaller portions. At the ultrastructural level, the macrophage is tightly aligned along the repair patch from its cytoplasm. This cell shows still extensive damage of the sarcomeres below the newly formed sarcolemma.

The patch from its cytoplasm. This cell shows still extensive damage of the sarcomeres below the newly formed sarcolemma. The repair patch is tightly squeezed between the healthy muscle at the ultrastructural level. Our data show that the repair patch is tightly squeezed between the injured and the neighboring cells. Moreover, the injured cell retained tight contact with the neighboring uninjured muscle tissue. In addition, the patch enclosed varying filamentous and membranous structures. The membranous profiles detected in the repair patch are heterogeneous in size and form with varying locations in the different repair patches examined. They may be accumulating material for resealing of the plasma membrane. Another interpretation is that these structures represent membranous debris entrapped by the precipitating repair proteins at the damaged area. It remains to be elucidated whether the amorphous regions of the patches have a structural organization as previously suggested by the ordered arrival of different repair proteins at the damaged site.

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The membrane repair processes have not been studied in the native tissue context of an intact, healthy muscle at the ultrastructural level. Our data show that the repair patch is tightly squeezed between the injured and the neighboring cells. Moreover, the injured cell retained tight contact with the neighboring uninjured muscle tissue. In addition, the patch enclosed varying filamentous and membranous structures. The membranous profiles detected in the repair patch are heterogeneous in size and form with varying locations in the different repair patches examined. They may be accumulating material for resealing of the plasma membrane. Another interpretation is that these structures represent membranous debris entrapped by the precipitating repair proteins at the damaged area. It remains to be elucidated whether the amorphous regions of the patches have a structural organization as previously suggested by the ordered arrival of different repair proteins at the lesion site. Detailed immunogold labeling experiments are needed to understand whether the repair patches contain structured assemblies of the different repair proteins.

Clearly the plasma membrane of the upper left myofiber in Fig. 4a has been restored again excluding the repair patch from its cytoplasm. This cell shows still extensive damage of the sarcomeres below the newly formed sarcolemma. Thus, the membrane of damaged myofibers seems to be repaired on the cell-proximal side of the patch. By real-time confocal imaging, we recently demonstrated that macrophages are required to remove the repair patch. The repair patch accumulates phosphatidylserine in a dysferlin-dependent manner. The latter serves as an "eat-me" signal for macrophages. The previous confocal real-time analysis showed that the macrophage takes up the repair patch in smaller portions. At the ultrastructural level, the macrophage is tightly aligned along the repair patch. This tight association of the macrophage may help maintaining the integrity of the barrier function while the macrophage removes pieces from the repair patch. The cytoplasm of the macrophage next to the repair patch was rich in various sized vesicles. These vesicles may be involved in phagocytosis of the repair patch.

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Methods

Zebrafish husbandry. Zebrafish husbandry and experimental procedures were performed in accordance with German animal protection regulations (Regierungspräsidium Karlsruhe, Germany, AZ35-91-18581/G-137/10). Publication of protocols and machines used was approved by the IGBMC authorities. Tg(kdrt/Hsa.HRAS-mCherry)916 was used to visualize blood vessels36.

Cloning, microinjection and myofiber damage. Cloning was carried out following standard procedures (supplementary information). Muscle expression of fusion constructs was driven by the unc45b promoter37. Approximately 100 eggs were used for each injection. The eggs were injected (DNA concentration of 20–80 ng/μl) between 1–2 cell stage with a drop of ~1/106 the size of the yolk using a glass injection needle. At 3–5 dpf embryos were embedded into 0.5% low melting point agarose to immobilize them onto a microscopy glass slide (detailed protocol see supplementary information). Muscle membrane lesions were introduced into single expressing muscle fibers as described previously18.

Cryofixation, freeze substitution and plastic embedding. Zebrafish embryos were cryofixed (vitrified) 15–30 min post membrane damage by high pressure freezing. The embryo was cut due to limitations of the carrier size and only the posterior half was high pressure frozen. After freeze substitution the samples were embedded into Lowicryl HM20 resin and blocks were polymerized using UV light (for detailed protocol see supplementary information).

Construction of the MIM. The MIM was constructed using the mechanical and optical workshops of the IGBMC. The MIM will be built by the IGBMC workshops for interested parties upon request. Interested laboratories can directly contact Didier Hentsch or Nicolas Lemercier.

Trimming and sectioning. Serial sections (250–260 nm thick) were prepared from the blocks using a UC7 ultramicrome (Leica, Austria) equipped with a Jumbo knife (Jumbo histo diamond knife 45°, Diatome, Switzerland) with a very large diamond boat hence facilitating collection and arrangement of the serial sections.

For the localization of the ROI in the block we used a method called Depth Evaluation of Axial Position of ROI (DEAP) to measure the distance of the ROI from the block surface (Fig. S2). To this end, a copper plain grid (50 μm single hole copper grids; Electron Microscopy Science, GA50-Cu) was deposited on the block surface with a micromanipulator (Fig. S2a) and the MIM was focused on the grid surface illuminated by reflected white light (Fig. S2b). The grid is then removed and the MIM is focused on the fluorescent ROI (Fig. S2c). The difference between these two positions is then calculated by taking into account both the grid thickness and the axial focus shift induced by the Lowicryl refractive index (Fig. S2d–f). With this information, the excess resin from the surface to slightly above the ROI can be removed safely in large steps followed by serial sectioning in a controlled way through the ROI.

Long ribbons were carefully collected and parked for immobilization in stretches perpendicularly to the diamond-boat with hair-filaments arranged in a ladder-like pattern38. The immobilized ribbons were scanned with the 10x objective of the MIM. Once the positive sections were identified, they were transferred onto EM-slot grids (2 × 1 mm slot formvar-coated EM grids; Agar Scientific, G2500PD). Once the sections of interest were secured onto EM grids, they were observed at higher magnification with the 40 × objective of the MIM and checked for the presence of a red dot (dysferlin accumulation at the lesion site) and green dot (accumulation of Annexin A2a) at the same site, and images were acquired. The coordinates of the lesion spot as well as those of the carrier size and only the posterior half was high pressure frozen.

The motorised stage then moved the grid to the right place (ROI) for tilt series acquisition. LM coordinates were recorded using Fiji or ImageJ software. The matrix was then used to calculate the TEM coordinates of the ROI (Fig. S3c,d). A coordinate converter GUI (Fig. S3e) allowed the users to enter the coordinates of the selected points and performed automatically the required calculation in a “user-friendly” way (Fig. S3e). The motorised stage then moved the grid to the right place (ROI) for tilt series acquisition.

Open source protection. Creative-Commons-MIM. The whole MIM assembly, the DEAP method and related devices described in the manuscript, the control software and the associated workflow are licensed under Creative Commons Attribution Non-Commercial Share-Alike license (CC-BY-NC-SA).

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Author Contributions
N.L., V.M., M.T., C.A., Ta.B., Ti.B. and U.S. designed the experiments. J.L.V. was scientific advisor. N.L., V.M., M.T., and C.A. performed experiments. N.L., D.H. and S.T. designed and built the MIM. N.L., C.A., V.M., P.S. and U.S. wrote the manuscript.

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