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A bioorthogonal chemistry approach to the study of biomolecules in their ultrastructural cellular context

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Citation

Elsland, D. M. van. (2018, January 11). *A bioorthogonal chemistry approach to the study of biomolecules in their ultrastructural cellular context*. Retrieved from <https://hdl.handle.net/1887/61131>

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Issue Date: 2018-01-11

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Electron Microscopy for Biochemical Research

The magnification power of an electron microscope (EM) can reach over a thousand times beyond that of a conventional light microscope and allows scientists to study specimens with nanometer scale resolution.¹ The EM is therefore the main tool for the imaging of cellular ultrastructures and has given rise to many observations that form the foundation of modern cell biology. Virtually all organelles and cell inclusions were either discovered or resolved in finer detail using the EM. These descriptions have laid the foundation for experimental manipulations directed at unravelling cell function and understanding how cellular structures vary in normal, experimental, and diseased states.

Electron microscopes

Generally two different types of electron microscopes are used for biological research; the scanning electron microscope (SEM) and the transmission electron microscope (TEM) (Figure 1). The SEM is commonly used to obtain three-dimensional ultrastructural information on the surface of individual cells or small

specimens and makes use of a low-energy electron beam (~ 0.2 keV to 40 keV) that scans the surface of a specimen. When this beam strikes the specimen, low energy secondary electrons are produced from the uppermost layers of the specimen, that are collected, processed, and eventually translated into an image.^{2,3}

The transmission electron microscope (TEM) transmits a high-energy electron beam (120-300 keV) through a specimen. The TEM can be used to study (the interior of) bacterial, mammalian and plant cells as well as viruses and proteins, and provides a two-dimensional image of the substructures that these specimens consist of.^{2,4}

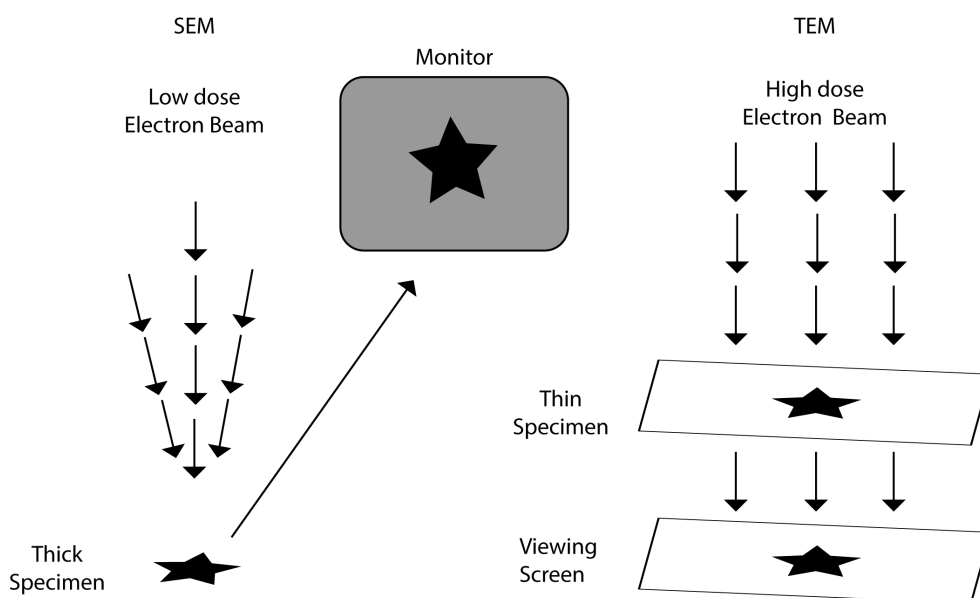


Figure 1: Basic principles of the transmission electron microscope (TEM) and the scanning electron microscope (SEM) (adapted from ²).

TEM sample preparation; fixation, dehydration, embedding, sectioning and staining

Biological samples cannot be imaged with the electron microscope whilst alive. The vacuum in the column of the EM -necessary for the coherent targeting of the electron beam- evaporates water in (living) biological samples and this causes damage to both the sample and the EM. Moreover, at the location where the (high-)energy electron beam hits the sample temperatures are elevated. Biological

samples must therefore be dehydrated prior to EM analysis. For small samples, such as purified proteins or virus particles, this can be done by air-drying or rapid freezing. However, larger biological samples need additional sample preparations, such as fixation and dehydration. Moreover, since with TEM the electron beam is transmitted through the sample, biological specimens such as mammalian cells need to be sectioned into ultrathin sections (30 to 150 nm), which requires hardening of the sample. Throughout the years many protocols have been developed to ensure the TEM analysis of biological samples and can be broadly grouped into; cryo-fixation strategies for cryo-EM analysis, whereby chemical fixation dehydration, plastic embedding and staining are obsolete; freeze-substitution strategies, whereby samples are first cryo-fixed after which frozen water is dissolved by organic solvents that contain chemical fixatives and chemical fixation strategies whereby samples are chemically fixed after which freezing or resin embedding can be performed. In the next part of this chapter the main used chemical fixation strategies will be discussed that can be used for the TEM analysis of mammalian cells.

Aldehyde fixation

Fixation is usually the first step of TEM sample preparation and protects the sample against disruption during embedding, sectioning and electron beam exposure. Generally the aldehydes glutaraldehyde and (para)formaldehyde are used for the (primary) fixation of a biological EM sample. Glutaraldehyde is a five-carbon compound that contains terminal aldehyde groups that primarily react with the amino groups of lysines (amino acids), and -to a lower extent- with lipids, carbohydrates, and nucleic acids (Figure 2A).² Formaldehyde forms methylene hydrate in aqueous solutions that primarily reacts with the side chains of amino acids and forms reactive hydroxymethyl side groups that can react with each other (Figure 2B).⁵ Methylene hydrate has the highest affinity for cysteine, lysine, histidine and tyrosine, but also reacts with nucleic acids and lipids.⁶

Aldehydes cross-link molecules adjacent to each other. For example, soluble proteins can cross-link to each other or to cytoskeletal or membrane-associated proteins, eventually resulting in a meshwork held together by a multitude of aldehyde molecules.² Glutaraldehyde cross-linking is irreversible and withstands acids, urea and heat, while paraformaldehyde cross-linking is reversible.² In case bridging of the hydroxymethylene groups has not yet occurred they can be rapidly

returned to their original groups if the paraformaldehyde is washed away. It is therefore that the cross-linking capacities of glutaraldehyde are stronger than those of paraformaldehyde.² Besides differences in their cross-linking capacities, the penetration rates of glutaraldehyde and paraformaldehyde also differ. Glutaraldehyde penetrates generally very poorly in compact tissues that have multiple membrane layers.⁷ Paraformaldehyde on the other hand penetrates about five times faster than glutaraldehyde.⁷ It is therefore that fixation protocols have been designed that combine both aldehyde fixatives. An example of such a protocol is the Karnovsky fixative.⁸ This fixation protocol utilises a relatively low percentage of formaldehyde that in theory penetrates fast and temporarily cross-links structures that are later more permanently stabilised by glutaraldehyde.^{2, 8}

Osmium tetroxide fixation

Osmium tetroxide contains four double-bonded oxygen molecules and works as a secondary fixative by reacting with lipid moieties (Figure 2C). Glutaraldehyde followed by osmium tetroxide is considered as a fixation protocol that is capable of stabilizing the maximum number of different cell components.² In addition to its fixation capacities, it is widely believed that the unsaturated bonds of fatty acids are oxidised by osmium tetroxide, which adds additional density and contrast to the biological sample when imaged with the TEM.⁹

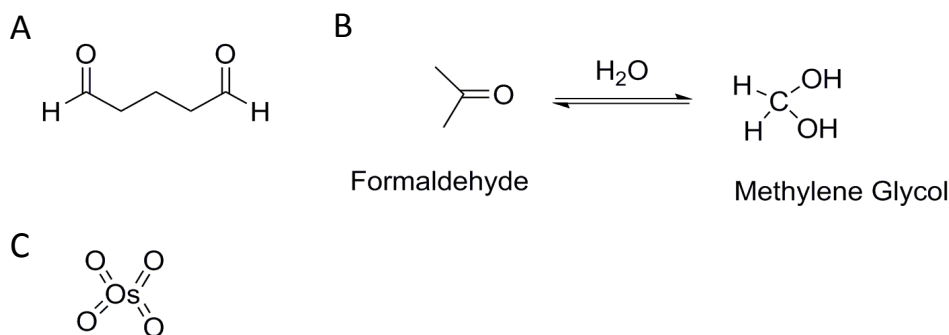


Figure 2: A) chemical structure of glutaraldehyde, B) chemical structures of formaldehyde and methylene glycol, C) chemical structure of Osmium tetroxide.

Dehydration and resin embedding

To cut ultrathin sections from relatively soft biological specimens, specimens need to be hardened. Conventionally this is done by infiltrating fixed and dehydrated specimens with a liquid plastic monomer that is subsequently polymerised *in situ*.

Dehydration is generally performed upon the gradual replacement of water by graded series of dehydrating agents such as ethanol or acetone. After dehydration the dehydrants are gradually replaced by resin monomers. These resin monomers are in the majority of instances epoxy resins, such as Epon¹⁰ and Araldite.^{11, 12} Epoxy resins are polyaryl ethers of glycerol bearing terminal epoxy groups (Figure 3A). To ensure resin polymerisation, three components must be included in the resin: (1) the epoxy resin monomer, (2) a hardening agent (for example; dodecenyl succinic anhydride (DDSA) (Figure 3B) or nadic methyl anhydride (NMA) (Figure 3C)), and (3) an accelerator (for example benzyldimethylamine (BDMA)) (Figure 3D). After resin infiltration, resins get cured at 60 °C. As an alternative to epoxy resins, acrylic embedding media such as Lowicryl can be used, which get cured at lower temperatures.^{2, 13}

Hardening the sample without resin; the Tokuyasu technique

TEM samples can also be hardened with rapid freezing. However, this has to occur fast and samples need to be cryo-protected in order to prevent ice-crystal formation. The most renowned method for freezing samples for TEM analysis is the Tokuyasu technique.¹⁴ With this strategy biological samples are first aldehyde fixed and embedded in gelatine, after which small sample blocks are cut and subjected to a sucrose infiltration step that functions as a cryo-protectant. Sample blocks are then placed on a metal specimen holder and are rapidly frozen and stored in liquid nitrogen.

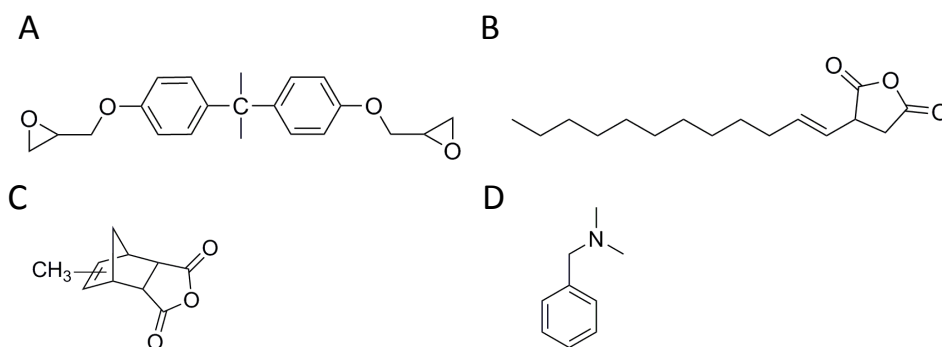


Figure 3: A) Idealised chemical structure of a typical epoxy, B) chemical structure of dodecenyl succinic anhydride (DDSA), C) chemical structure of nadic methyl anhydride (NMA), D) chemical structure of benzyldimethylamine (BDMA).

Sectioning

Once a sample is hardened by one of the above methods, ultrathin sections can be obtained. Sectioning of resin embedded samples is performed using a diamond knife and an ultramicrotome, and generally involves the following steps; trimming or shaping of the specimen block with a glass or diamond knife, cutting of sample sections in an ultramicrotome with a diamond knife, retrieving sections and placing sections onto a specimen grid holder.² Sections can vary in size from 30 to 150 nm. However, the general rule is the thinner the section, the higher the resolution.² Sectioning of frozen samples is known as cryo-ultramicrotomy and works along the same principle, with the exception that sectioning is performed at -80°C to -140°C and frozen sections are retrieved with a sucrose droplet.¹⁴

Staining

EM images consist of various shades of grey that represent the density differences in a specimen; darker shades are areas of the specimen that have greater density, whereas brighter areas of the specimen have less density. Since unstained biological samples have little density differences it is important to increase the image contrast by reacting cellular components with heavy metals.² Contrast enhancement can be established with the secondary fixative osmium tetroxide (Figure 2C)¹⁵, and with the heavy metal stains lead citrate and uranyl acetate (Figure 4A/B). Osmium reacts with the lipid moieties of a specimen and the lead ions of lead citrate bind to negatively charged components such as carboxylate groups and osmium-reacted areas. Lead citrate staining is therefore commonly performed on sample sections that have been osmium-fixed and resin-embedded. Uranyl ions react with phosphate and amino groups, thereby staining nucleic acids and certain proteins, and are generally used to stain rehydrated cryo-sections.^{16, 17}

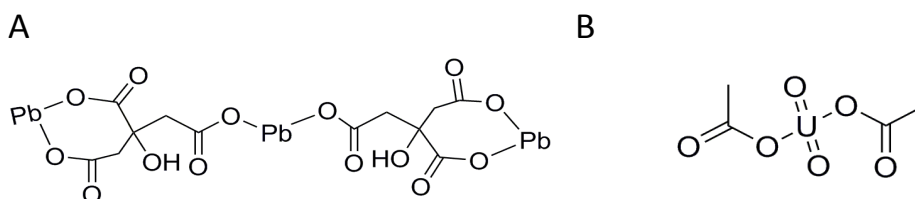


Figure 4: A) chemical structure of lead citrate, B) chemical structure of uranyl acetate.

Biomolecule detection with electron microscopy

Although TEM enables observations at nanometer-scale resolution, the revealed ultrastructures remain uncharacterised. It is therefore that several techniques have been developed to label the biomolecules that are present in the EM-revealed structures. Most commonly colloidal gold particles are used for this purpose.¹⁸⁻²¹ These particles can be functionalised with macromolecules used in immunocytochemistry, such as antibodies and protein A,¹⁸ and give a punctate and precise labelling pattern of the biomolecule of interest (Figure 5).^{18, 19} Gold labelling is generally performed on Tokuyasu cryo-sections as with this technique epitopes remain preserved and accessible to the functionalised gold particles.^{14, 22, 23}

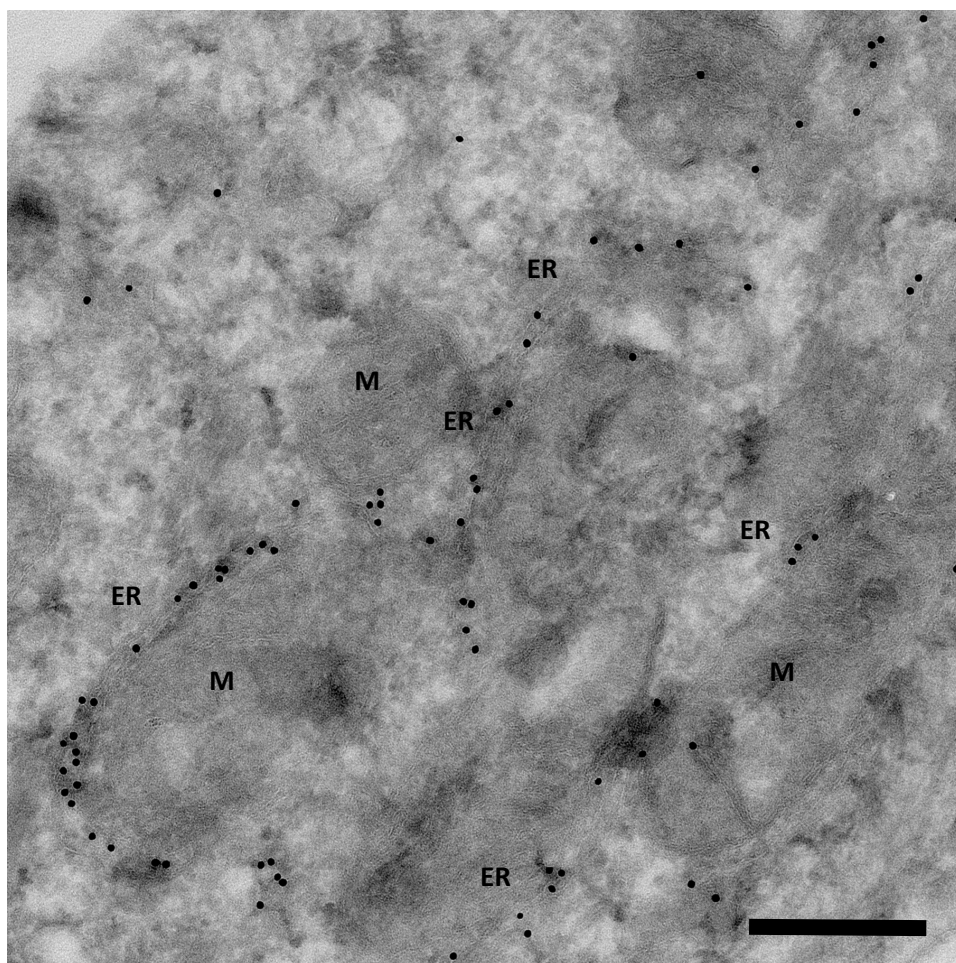


Figure 5: Example of 15 nm immunogold labelling of the endoplasmic reticulum, Endoplasmic Reticulum (ER), Mitochondria (M) (*Figure example from author's own data collection*).

Besides gold markers oxidizing substrates, such as diamino-benzidine (DAB), are also often used to generate localised osmiophilic precipitates which can serve as electron dense markers.²⁴ These reporter precipitates can be formed using photosensitizing dyes^{25, 26}, peroxidases such as horseradish peroxidase (HRP)²⁴ and engineered peroxidase enzymes, such as APEX/APEX2.²⁷ By conjugating the photosensitizing dyes/enzymes to specific antibodies or ligands, this approach can be used to visualise specific proteins.^{28, 29} Due to the high penetrability of DAB it can be performed prior to sectioning. Moreover, since DAB precipitates are not affected by resin embedding, the DAB-labelling approach is commonly applied to resin-embedded samples which are generally not susceptible to any on-section labelling strategies.²

Correlative Light and Electron Microscopy approaches

Fluorescent markers can also be used for biomolecule detection in case fluorescent microscopy (FM) is combined with EM.³⁰ Specific biomolecules and cellular structures can be identified -upon use of a fluorescent marker- with FM, after which ultrastructural information about their subcellular location and context can be obtained with the EM.³⁰ As a result, one can interpret the fluorescent labelling on a complete cell, whereas electron dense markers are too small for detection at a low magnification. Over the years many of these correlative light and electron microscopy (CLEM) strategies have been reported,³⁰ which can generally be divided into these two: the ones that combine live-cell FM imaging with EM imaging, and those that combine FM and EM imaging of the same sample sections.

Correlating live-cell dynamics with EM ultrastructures

When combining live-cell FM imaging with EM imaging, dynamic processes observed with FM can be analysed with ultrastructural detail using the EM.³⁰ Live-cell CLEM imaging methods basically consist of six steps: 1) the incorporation of a fluorescent marker in live cells, 2) live-cell FM imaging, 3) sample preparation for EM analysis, 4) ultramicrotomy, 5) EM imaging, 6) correlation of FM and EM images.³¹ An example of this workflow is illustrated in Figure 6. One of the first attempts of combining live-cell FM with EM, has been reported by Nakata et al.³² In this study the subsequent EM imaging of *in vivo* imaged GFP-tagged plasma membrane proteins was demonstrated. Although this strategy attempted to image the same cell with both imaging modalities, it did not allow for the

identification of the very same cell or region of interest (ROI).³² This problem relies in the fact that EM imaging is in need of extra sample preparations and ultrathin sectioning; it is therefore very difficult to retrace the originally live-cell FM-imaged location with the EM. To this end various methods have been reported that focus on the EM retrieval of a ROI that has been imaged with live-cell microscopy. For example Van Rijnsoever et al. reported the retrieval of a ROI by using gridded coverslips.³³

Correlating LM and EM from the same sections

Although strategies have been reported that improve the retrieval of the ROI in live cells with EM, it remains problematic to retrieve the exact ROI. It is therefore that strategies have been developed to correlate FM and EM images of the same sample section, a strategy referred to as on-section CLEM. This strategy does not allow for live-cell imaging, but does ensure that the exact same ROI is imaged with both imaging modalities. The on-section CLEM workflows generally consist of the following six steps: 1) the labelling with of a fluorescent marker in live, fixed or sectioned cells, 2) sample preparation for EM analysis, 3) cryo-ultramicrotomy, 4) FM imaging, 5) EM imaging, 6) correlation of FM and EM images. An example of this workflow is illustrated in Figure 7. The main difference between live-cell CLEM and on-section CLEM is that the fluorescence labelling can occur at different stages. For example, live or fixed cells can be labelled with a fluorescent reporter that remains fluorescent after EM sample preparation and ultrathin sectioning.³⁴ Another approach is to label the specimen after embedding and ultrathin sectioning.³⁵ This can also be applied in sequence to specimens that already contain fluorescence labelling, and thus allows for additional labelling steps after sample preparation. In general the on-section CLEM strategy is very useful to use FM as a map to guide through the sectioned sample, to identify transiently transfected cells, and to identify rare structures of interest. Moreover ultrathin sections (typically 50-80 nm) yield very sharp fluorescence images that lack the z-axis blur.³⁶ Correlation of LM and FM in case of on-section CLEM is facilitated by the use of fluorescent/electron-dense fiducials. These fiducials can be imaged with both imaging strategies and are used as a reference during correlation.³⁵

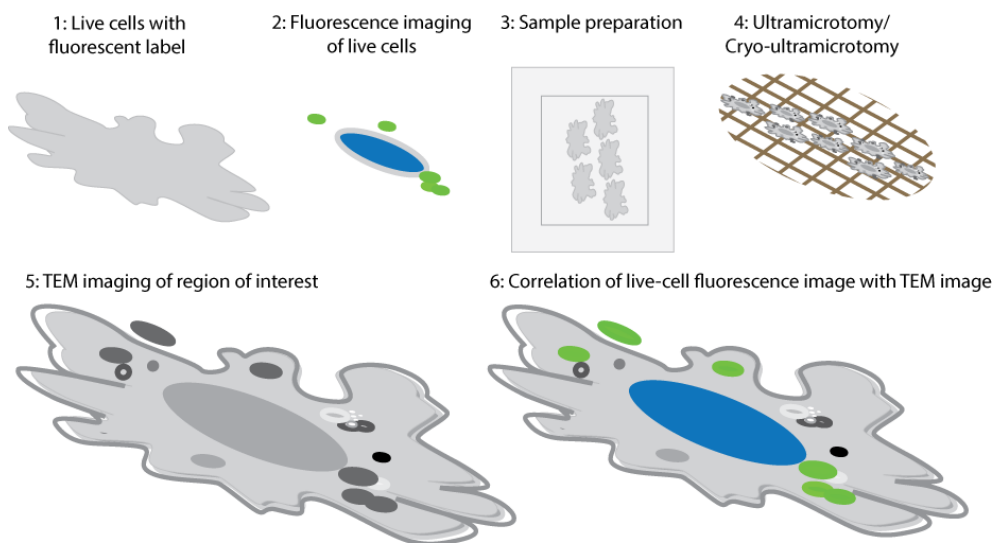


Figure 6: Schematic representation of a CLEM workflow that combines live-cell imaging with EM.

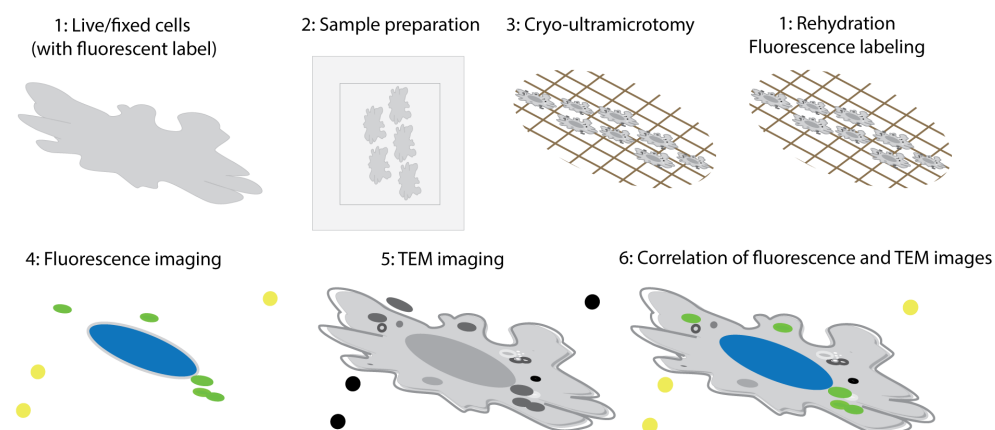


Figure 7: Schematic representation of an on-section CLEM workflow.

Conclusion

To ensure the TEM analysis of large biological samples, samples need to be fixed, dehydrated, hardened, sectioned and stained. These procedures can be varied upon the research questions to be answered, and as a result many protocols have been developed. For example, research questions in need of the best possible ultrastructural preservation usually involve vigorous cross-linking with glutaraldehyde and resin embedding, whereas mild aldehyde fixation and cryo-

sectioning is performed when (immuno-)labelling of the EM-revealed structures is desired.

Labelling of biological samples for TEM analysis is traditionally performed with electron-dense markers such as colloidal gold or DAB. Alternatively, specimens are imaged with both FM and the EM, followed by correlation of the acquired data sets. This strategy referred to as CLEM is especially useful for the localisation of rare events, and can be applied in combination with a wide variety of fluorescent labelling techniques.

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