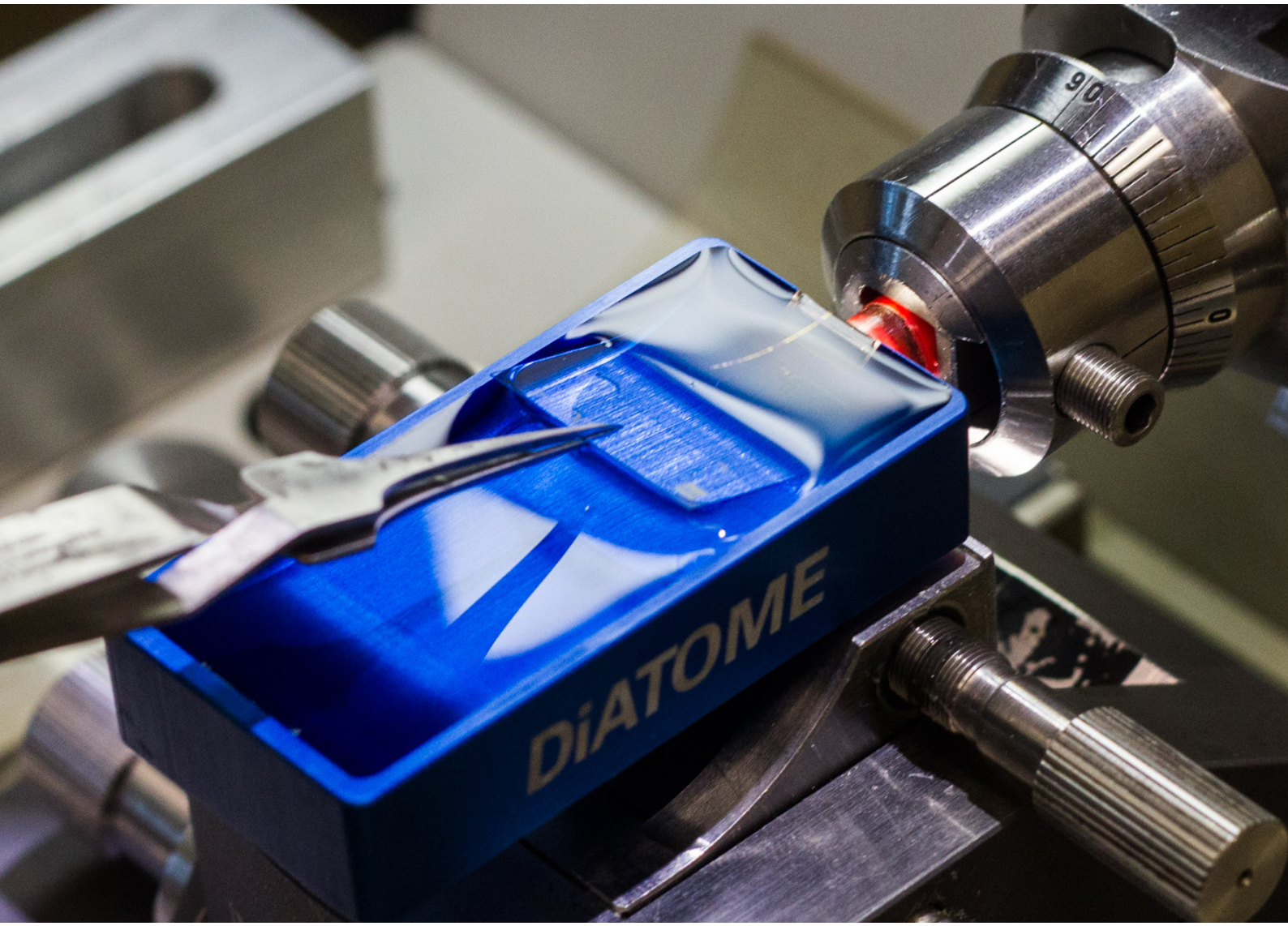
Technical Note



Sample Preparation for Correlative Array Tomography

ZEISS

Seeing beyond

Sample Preparation for Correlative Array Tomography

Authors: Eric Hummel, Kirk Czymmek,
Ralph Neujahr, Alexandra Elli
Carl Zeiss Microscopy GmbH, Germany

In array tomography ordered, ribbon-like assemblies of ultrathin serial sections are deposited on a solid substrate and imaged afterwards. The resulting images are then aligned and reconstructed into a three-dimensional representation of the object. Depending on the preparation and labelling regime, different imaging modalities can be applied. When using light microscopy, the labelling with fluorescent markers would be the obvious choice, whereas the imaging in a scanning electron microscope would require impregnation with heavy metals. Depending on preparative constraints, the combination of diverse imaging modalities or truly correlative imaging is possible. The integrated ZEN CAT module enables scientist to automatically identify serial sections and specific regions of interest in light and electron microscopy. After registration and alignment routine implemented in the CAT module direct correlation of the light and electron microscopy datasets can be drawn.

Introduction

The term AT was first coined by Stephen Smith (Micheva& Smith, 2007) and then developed further and used for proteomic imaging of neuronal circuits by the same lab (Micheva et al., 2010). A number of similar or related methods have been published recently, which differ in a variety of ways from the original method. Sample preparation is the first essential step for correlative array tomography. Beside the generation of section ribbons maintenance of fluorescence and sufficient contrast for SEM imaging is the second constrain for successful correlative array tomography. The method of correlative array tomography was so far mainly used for combined light and transmission electron microscopy approaches. The limited size of TEM carriers limits the number and size of the applied section to the size of traditional TEM Grid. Using new methods such as our ZEISS SEM portfolio allows to collect sections with larger surface and is in numbers not limited to the size of TEM Grids. In the following we want to describe approaches how to achieve optimum sample preparation for our new correlative approach. Kukulski et al (2010) have shown preservation of fluorescence during the whole embedding process using low temperature embedding in Lowicryl resin. Disadvantage of the described method is the difference of maintenance of fluorescence in different fluorophores. In our approach we are following the workflow which is recently described in Wacker and Schroeder (2013) which uses immunolabelling approaches to provide the specific fluorescence signal. This methods provides specific signal in high pressure frozen and freeze substituted samples as well as in chemical fixed cells and tissues embedded in methcrylate based resins.

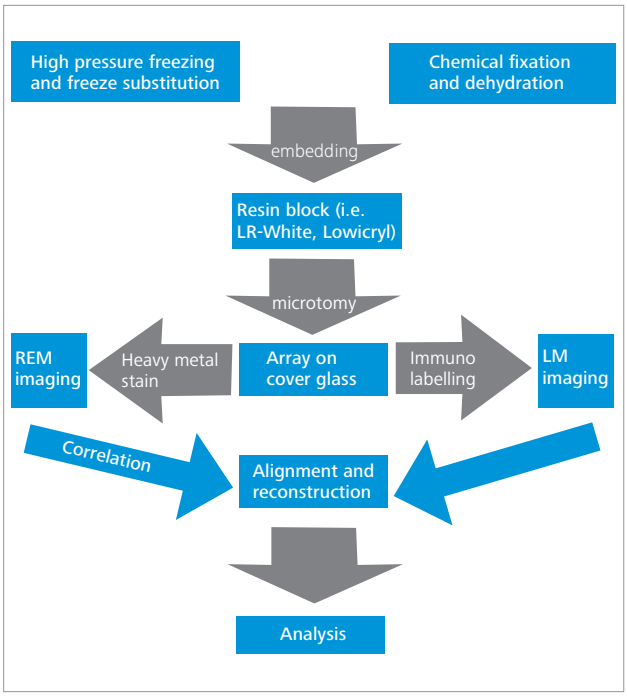


Figure 1: *Workflow description correlative array tomography for best possible light and electron microscopy analysis (adapted from Wacker & Schroeder 2013)*

2. Methods and Results

2.1. Fixation and Embedding

Fluorescence imaging in embedded samples could be distinguished into the following categories:

Postembedding Immunoprocedure

Immunolabelling procedures can be either praeembedding labellings (antibodies/stains are applied before embedding) or postembedding (antibodies/stains are applied to ultrathin sections). As known for all approaches implementing immuno labeling procedure the choice of the fixation method and embedding is crucial. Chemical fixation methods should be carried in general under low temperature conditions, start with a fixation and dehydration at 4° C and resin substitution and embedding at -20° C using either LR-White or Lowicryl based resins.

The best available fixation method so far is high pressure freezing followed by freeze substitution and embedding at low temperature. Rapid freezing provide optimal sample preservation and preservation of proteins and their related epitopes. Compared to chemical fixation high pressure freezing provides best labeling results. In chemical fixation routines aldehydes could influence protein structure which affects immuno labeling results. The table below shows a general protocol for embedding chemical fixed cells:

Step	Temperature	Time
Fixation	4°C	1 h
3x Washes in Buffer	4°C	10 min each
3x washes in H_2O	4°C	10 min each
Postfixation in 0.5% to 1% Uranylacetat	4°C	Over night
Washes 3x in water	4°C	10 min each
Dehydration with Ethanol (10 %, 30%)	4°C	30 min each
Dehydration with Ethanol (50%, 70%, 90%)	-20°C	30 min each
Resin infiltration LR-White (25%, 50%, 75%, 3x100%)	-20°C	30 min each
Embedding in capsules UV light	-20°C	2 days

The Samples are subsequently sectioned and labelled with classical immunolabelling protocols after embedding (see chapter labelling techniques).

Preembedding Immunoprocedure

When cells are antibody labelled before embedding, the labelling efficiencies drop considerably once the specimens become thicker. It has been shown that labelling efficiency decreases, especially deeper inside the tissue, which is because of lower antibody penetration capacity – e.g. more than several micrometers is rarely achieved. Cell cultures and single cells are generally suited for praeembedding techniques. The preembeding process involves classical immunofluorescence routines. Classically immunofluorescence uses sodium sodiumborhydrate to quench fluorescence of the aldehydes. Borhydrate can influence the cell ultrastructure dramatically, for correlative low concentrations or alternative routes are recommended. Glycin for examples could be used to quench Glutaraldehyde fluorescence or mixture of PFA with low concentrations of fixative could be used (GA not higher than 1%). After fixation and several washes, the protocol starts with classical blocking steps followed by varying permeabilization steps (see for discussion for the ideal method Humbel et al (1998)) Preferred method is permeabilization with detergents such as Triton X100 or Brij58 in very low concentrations (not higher than 0.1%). After a classical Alexa Fluor labelling the embedding has to be carried out as described above at low temperatures. The best resin to maintain antibody signal during the whole process seems to be Lowicryl resin, embedding temperature -35°C. The polymerized samples should be stored in a cool and dark place and the sections should be used the same days for immunofluorescence.

Preservation of Fluorescence

New protocols allow to identify specific fluorescent patterns directly in the EM sample after preparation (Sartori et al., 2007; Nixon et al., 2009; Watanabe et al., 2011). Such preserved fluorescent protein signals in high pressure frozen EM samples could be directly correlated with three-dimensional (3D) electron tomographic data as shown by Kukulski et al. (2011). In this study, the preserved GFP signals allowed the targeting of ultrastructural features like HIV particles and microtubule endstructures with high precision in 300 nm Lowicryl sections (Höhn et al 2015)

A quite efficient protocol is for maintaining fluorescence in LR-Gold resin was suggest by Höhn et al (2015): Cells were grown on glow-discharged, carbon-coated and Poly-L-Lysin covered 160 µm thick sapphire discs. Cells were subsequently high pressure frozen after growing on sapphire discs. A coordinate system is imprinted on the surface to facilitate orientation on the sapphire discs and to provide a reference for different imaging modes. The samples were further processed by freeze substitution in a temperature-controlling device with a substitution medium consisting of acetone supplemented with 0.1% uranyl acetate and 5% water.

The temperature was gradually raised from –90 to –20 °C over a 16 h period. After washing the samples three times with 100% acetone at –20 °C, the cells were infiltrated with increasing concentrations (25, 50, 75 and 100%;1h each) of LR-Gold in acetone. The final embedding in 100% LR-Gold occurred over 12 h and samples were UV polymerized starting at –20 °C for 24 h, after which the temperature was raised to 20 °C over 4 h and UV polymerization continued for 24 h. Compared to the so far mostly used Lowicryl protocols (Kukulski et al 2010) LR Gold resin is easier to handle.

2.2. Serial Sectioning

In the last chapter we have proven that correlative array tomography is feasible using typical embedding procedures. We have described three mayor embedding routines suited for correlative array tomography. Nevertheless the most important steps to gain large ribbons of sections for correlative array tomography are trimming, sectioning and the ribbon collection on glass slides. Here we describe the subsequent steps which allow the production of large ribbons.

Prepare Cover Slips

Before starting cutting the selection of the adequate substrate is of mayor importance for the successful workflow. We recommend to use ITO coverslips with marks (Cover glasses compatible with ZEISS specimen holder CorrMic Life Sciences for cover glasses 22 x 22 mm item number 432335-9300-000), a thin Ito layer allows imaging on LM and EM without additional coating steps. Preassigned marks on the coverslips allow an easy fast forward calibration and keep the full flexibility for all later processing steps within the workflow.

Especially for longer sections ribbons we recommend to coat the ITO slips before use with Poli-L-Lysine (0.1 mg/ml) (Sigma ordernr. P7280) to allow the sections to adhere. There dip the ITO coverslips into the Poli-L-Lysine solution and let it try before use.

Trimming

Trimming the sectioning area as accurate as possible, with the leading and trailing edges of the block being parallel, is essential. The sample should be placed in the holder only once and not be removed until serial section production is finished. Best is to choose the angle at 0° an keep it there. Best possible method for trimming is using a diamond trimming knife (Diatome Inc. Biel) and use trimming steps of 1 µm. The sides of the trapezoid are trimmed at 49°.
For a perfect edge shortly before end the side trimming some sections at 47° can be applied (around 5 µm).

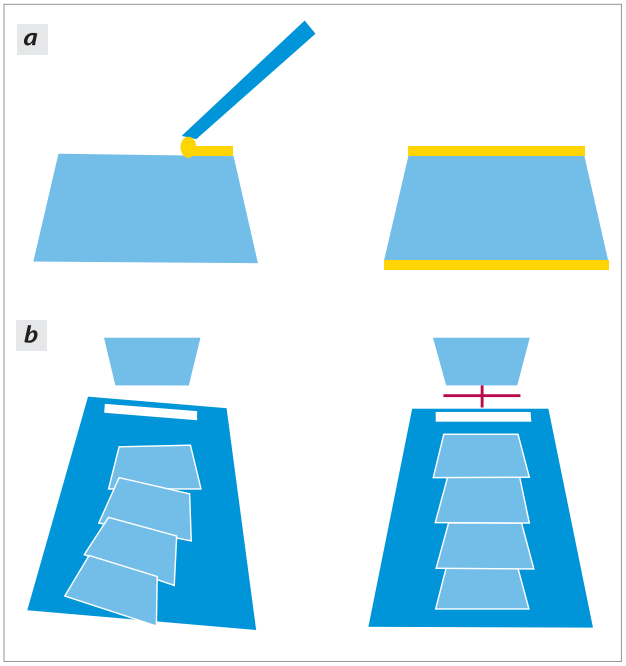


Figure 2: *Precutting adjustments a: Superglue is placed with a tooth stick on the cutting edges to allow to produce longer ribbons. b: The right orientation of the cutting and the knife surface is essential for perfect straight orientation of the ribbons. The knife / the cutting surface have to be absolutely in parallel.*

Sectioning

Adding glue on the leading and trailing faces of the pyramid helps to keep long ribbons together throughout the handling process. A second possibility is the use of the deionizer on the microtome. Tension applied depends on the resin used. Using the deionizer provides long ribbons without using glue. It is useful to place the charged ITO coverslips for 30 seconds under the deionizer. Charging of ITO slides makes it difficult to pick the serial ribbons without disturbance.

For successful transfer of long section ribbon to ITO cover slips a diamond knife with a Jumbo boat (Diatome Inc., Biel, Switzerland) is a very useful tool (3 A). The jumbo boat allows to mount a whole glass slide below the water surface. We typically suggest 22 mm ITO cover slips with applied L-marks (Cover glasses compatible with ZEISS specimen holder CorrMic Life Sciences for cover glasses 22 x 22 mm item number 432335-9300-000), these cover glasses allow full flexibility for the later workflow. The boat allows also to place whole coverslips and collect even longer ribbons (50 mm length; Micheva et al 2007). A micromanipulator (Fig 1B) is used to hold glass below the water surface and allow easier removal. Tension of the water surface could cause disturbances during removal of the slide. Some people add low concentrations of Triton (0.05%) into the knife boat to minimize that effect. After the prior steps the section process starts and acquires

long ribbons (the speed of the microtome depends always a bit on the sample we recommend 0.8 mm; Fig 3 c+d). During the whole section process the cover glass is hold in place until the whole cutting process is finished (Fig 3 e). The section ribbons are carefully oriented above the glass slide with a mounted hair (Fig 3 f). The glass slide is slowly retracted from the water (Fig 3 g). During the retraction process the ribbons get reorientated with the hair (Fig 3 h). A heat pen could be placed above the water surface to allow the ribbons to strengthen (minimization of folds in the ribbons (Fig 3 i). Chloroform which is typically used to strengthen Epon sections should not be used for LR-White and LR cold, the vapor destroys the sections.

Some groups also use dionizers to strengthen sections. The first sections of the ribbons are fixed on the glass at the water rims (Fig 3 j). After attached on the glass the coverslip slowly retracted to adhere the complete ribbons on the glass. After removal of the water the cover slips are tried on heat plate (30°C) and get prepared for further processing.

When mounting the sections on the substrate, a 'third hand' assistance might be particularly useful. This can be any kind of device, holding the substrate and allowing its slow, controlled removal from the knife boat (Micheva et al 2007). We recommend micromanipulators such as the ASH (RMC).

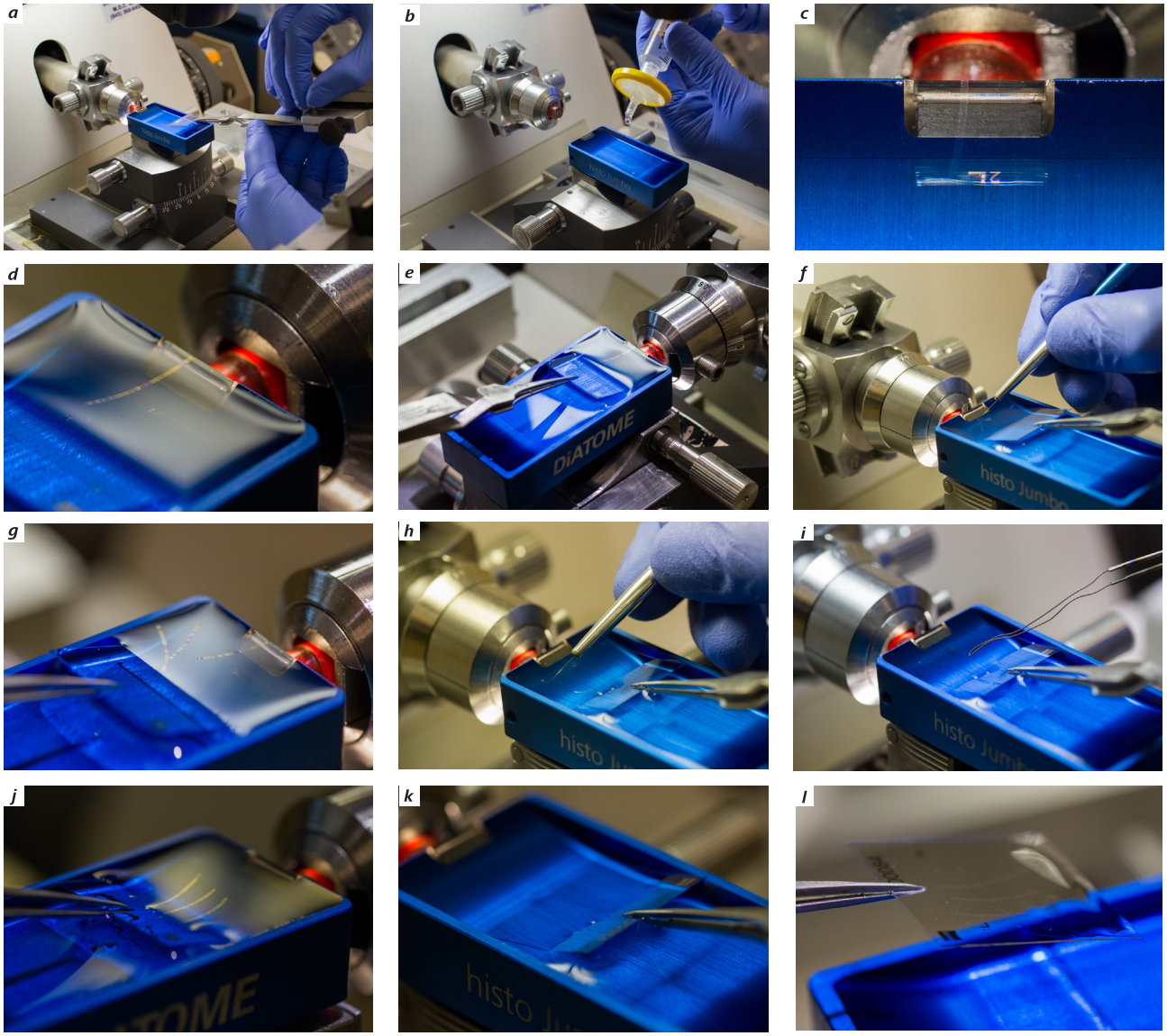


Figure 3: *Cutting workflow for Correlative array tomography a: Knife and waterlevel are adjusted and aligned. Before the cover glass is placed below water surface and moved towards the knife. b: Fine adjustment of cover glass and production of first sections. c: First sections ribbons are produce d: Sections ribbons grow until the length of the cover glass would be covered. d: Stop cutting process. e-f: Arrange sections with a hair at the edge of the water surface. g: Slowly retract and move up the cover glass using the micromanipulator. h: Rearrange ribbons with hair on the water edge and let them attach on cover glass i: A hot needle can be used to stretch the sections j: Retract the cover glass slowly. k: Increase angle and move cover glass up out of the water: l: Cover glass out of water*

2.3. Immunolabelling (Postembeding)

The most intriguing workflow for correlative array tomography are immunolabelling procedures using immunofluorescence protocols. Smith and colleagues (Micheva el al 2010) managed to label with up to 10 different antibodies using antibody reelution protocols. For a detailed protocol of how to do that we recommend the publications from Steven Smith and colleagues. In this chapter we want to provide a standard immunolabelling protocol for immunofluorescence and want to focus on the specific requirements of cover glases. Typically thin sections are labelled on EM grids. Here we use coverslips instead of immunogold antibodies we apply Alexa fluor antibodies for correlative array tomography. The cover glasses are processed before starting the immunolabelling routine. We recommend to use a Grease pen to surround the areas where sections are placed (Kay et al 2013). This minimizes the amount of precious antibodies needed for the labelling routine. Be aware of the fact that grease pens are rather delicate for the electron microscope before transferring the sections to the EM a few washes with 50% ethanol should be carried out. The immunolabelling routine follows the classical immunofluorescence protocol:

Step	Chemical	Time
Blocking	1% BSAc in PBS, 0.5% fish gelatine, 1% goat serum	30 min
	0.1 % BSAc in PBS, 1% Glycine	15 min
Washes	0.1 % BSAc in PBS 3x	15 min each
Primary antibody	Concentration applies to antibody in 0.1% BSAc in PBS	90 min
Washes	0.1 % BSAc in PBS 3x	15 min each
Secondary antibody	1:200 for i.e.Alexa fluor 488 in 0.1% BSAc in PBS	90 min
Washes	3x in 0.1 % BSAc in PBS	10 min each

After the labelling processing Array tomography on the Light microscope is started.

2.5. Poststaining

Due to the lack of heavy metals during classical immunolabelling protocols poststaining is most of the time associated prior to the EM imaging step. Classical poststain routines such as a combined uranyl acetate/ lead citrate stain is ideally suited to produce the contrast needed for SEM imaging. Micheva et al (2010) tend to use Potassium permanganate as additional staining agent before adding uranylacetate and lead citrate. Washing steps with water between the different staining procedures are important. The time needed for the different steps differs from sample to sample (Uranylacetate from 10-30 min; Lead citrate 10-30 min; Potassium permanganate 30 sec-5min).

2.6. Electron Microscopy

After poststaining the samples are inserted into the SEM. The CAT software follows the same routine as already carried out for light microscopy. After a short 3 point calibration of the holder. The datasets from LM imaging are opened. The system automatically aquires the selected regions of interest (see product info for detailed description). After the acquisitions overlays are generated by doing a fine alignment

3. Discussion

FIB SEM tomography and serial blockface imaging (3view) are destructive techniques for the generation of 3D data out of the SEM. Array tomography is a preserving technique and allows multiple labelling with antibodies on the same sample. The Array tomography volume is similar to the other two techniques reconstructed from the serial slices imaged on the light and the electron microscope. (AT) involves reconstruction of images acquired from arrays of serial ultrathin sections. Unfortunately, conflicting requirements for preservation of immunoreactivity and ultrastructure severely limit attempts to combine IF-AT and SEM-AT on the same specimens (Micheva et al., 2010; Oberti et al., 2011). Manual acquisition of these volumes are tedious and increase bleaching effects. An automated and efficient software solution is needed to combine the advantages of light microscopy imaging and the ultrastructural information on the scanning electron microscope on deep z level information to achieve optimal and quick results.

With our new solution CAT (Correlative Array Tomography) we further want to overcome the hints of array tomography and provide an efficient solution of automated imaging both on LM and EM. Furthermore we offer alignment tools to generate overlays of the generated datasets and analyze this data in a single software. On hand we provide a efficient acquisitions due to the automated processes within CAT and as well on the other hand provide tools to register the datasets with each other.

References:

Hayworth, K.J., Kasthuri, N., Schalek, R. & Lichtman, J.W. (2006) Automating the collection of ultrathin serial sections for large volumeTEM reconstructions. Microsc. Microanal. 12 (Supp 2), 86–87.

Höhn, K., Fuchs , J., A. Fröber, A., Kirmse, R., Glass, M., Anders Österwein, M., Walther, P. Kräusslich, H. G., Dietrich, C. (2015). Preservation of protein fluorescence in embedded human dendriticcells for targeted 3D light and electron microscopy, Journal of microscopy 259, 121-128.

Horstmann, H., Körber, C., Sätzler, K., Aydin, D. & Kuner, T. (2012) Serial section scanning electron microscopy (S3EM) on silicon wafers for ultra-structural volume imaging of cells and tissues. PLoS One. 7,e35172.

Humbel, B.M., De Jong, M.D.M, Müller, W.H, Verkleij, A.J. (1998), Pre embedding Immunolabeling for electron microscopy: en evaluation of permeabilization methods and markers. Microscopy Research and Technique, 42, 43-58.

Kay, R., Smith, C., Wright, A. K., Serrano-Pozzo, A., Pooler, A.M., Koffie, R., Bastins, M.E., Bak, T., Abrahams, S., Kopeikina, K., McGuone, D., Frosch, M., Gillingwater, T.H:, Hyman, B., Spires-Jones, T. L. Studying synapses in human brain with array tomography and electron microscopy. J. Neurosci. 31, 16125–16138.

Lucas, M.S.,Günthert,M., Gasser, P., Lucas, F.,Wepf, R. (2012) Bridging Microscopes: 3D Correlative Light and Scanning Electron Microscopy of Complex Biological Structures. Method Cell Biol. 111, 325–356.

Kukulski, W., Schorb, M., Welsch, S., Picco, A., Kaksonen, M. & Briggs, J.A. (2011) Correlated fluorescence and 3D electron microscopy with high sensitivity and spatial precision. J. Cell Biol. 192, 111–119.

Micheva, K.D. Smith, S.J. (2007) Array tomography: a new tool for imaging the molecular architecture and ultrastructure of neural circuits, Neuron 55, 25–36.

Micheva, K.D., Busse, B., Weiler, N.C., O'Rourke, N., Stephen J. & Smith, S.J. (2010) Single-synapse analysis of a diverse synapse population: proteomic imaging methods and markers. Neuron 68, 639–653.

Micheva, K.D., O'Rourke, N., Busse, B. & Smith, S.J. (2010) Array tomography: high-resolution three-dimensional immunofluorescence. Cold Spring Harb. Protoc. 2010, 1214–1218.

Nanguneri, S., Flottmann, B., Horstmann, H., Heilemann, M. & Kuner, T. (2012) Three dimensional, tomographic super-resolution fluorescence imaging of serially sectioned thick samples. PLoS One. 7, e38098.

Nixon, S.J., Webb, R.I., Floetenmeyer, M., Schieber, N., Lo, H.P. & Parton, R.G. (2009) A single method for cryofixation and correlative light, electron microscopy and tomography of zebrafish embryos. Traffic 10, 131–136.

Oberti, D., Kirschmann, M.A.&Hahnloser, R.H.R. (2011) Projection neuron circuits resolved using correlative array tomography. Front Neurosci. 5, 50.

Reichelt, M., Joubert, L., Perrino, J., Koh, A.L., Phanwar, I. & Arvin, A.M. (2012) 3D reconstruction of VZV infected cell nuclei and PML nuclear cages by serial section array scanning electronmicroscopy and electron tomography. PLoS Pathog. 8, e1002740.

Sartori, A., Gatz, R., Beck, F., Rigort, A., Baumeister, W. & Plitzko, J.M. (2007) Correlative microscopy: bridging the gap between fluorescence light microscopy and cryo-electron tomography. J. Struct. Biol. 160, 135–145.

Schindelin, J., Arganda-Carreras, I., Frise, E., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Meth. 9: 676–682.

Watanabe, S., Punge, A., Hollopeter, G.,Willig, K.I., Hobson, R.J., Davis, M.W., Hell, S.W. & Jorgensen, E.M. (2011) Protein localization in electron micrographs using fluorescence nanoscopy. Nat. Methods 8, 80–84.

Carl Zeiss Microscopy GmbH
07745 Jena, Germany
microscopy@zeiss.com
www.zeiss.com/microscopy