



Correlative Microscopy Protocols

A Reference Guide to Correlative Sample Preparation



Seeing beyond

Cover image – FE-SEM & superresolution light microscopy on ultrathin yeast sections imaged with ELYRA and AURIGA CrossBeam: Correlation of GPCR localization to specific organelles in Yeast in order to understand heterologous protein trafficking. (Caplan J., Young C., Ross J., Jacobs S., Modla S, University of Delaware and Czymbek K. Carl Zeiss Microscopy, LLC.)

[For more details see White Paper “Correlative Protein Localization in Yeast”; High-Resolution Localization of Fluorescent Proteins Using Shuttle & Find for Superresolution and Scanning Electron Microscopy.]

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Preface

The quest to understand the complex interactions of structure and function in biology continues to fuel efforts in advancing correlative methods combining light and electron microscopy imaging.

With the availability of dedicated product solutions such as Shuttle & Find that provide an easy link between imaging modalities, the challenge for scientists is increasing to provide preparation protocols that are applicable to the specific sample of interest. Here the ultimate goal of any protocol is to retain a maximum of image information from both light and electron microscopy – essentially balancing the trade-off between preserving fluorescent signal for LM and obtaining EM contrast, e.g. by heavy metal staining of resin embedded samples.

Traditionally transmission electron microscopy has been well-suited for biological EM imaging. However, modern research increasingly relies on using state of the art field emission scanning electron microscopes (FE-SEM) for 3D EM imaging. While the latter provide comparable image quality for resin embedded samples as the traditional TEM they also provide

the benefits of ease-of-use and automation, thus significantly shortening time to result. In combination with serial section imaging (ATLAS) [1], in-situ ultramicrotomy (3View®) [2] or focused ion beam nanotomography (CrossBeam) [3] they effectively open up the 3rd dimension for advanced biological research.

The intention of this document is to provide an overview of existing sample preparation know-how for correlative microscopy. While many of the cited protocols still reference to TEM they typically are applicable to the modern technique of field emission SEM with little or no modifications.

Note

Unless otherwise mentioned, all protocols are published and citations are included.

Important: This document is not intended to provide a stand-alone “how to” guide or “cook book”. Readers are obliged to refer to detailed protocol descriptions especially for all aspects of personal safety within the original literature.

References:

- [1] Kuwajima M., Mendenhall J. M., Lindsey L. F., Harris K. M. (2013): Automated Transmission-Mode Scanning Electron Microscopy (tSEM) for Large Volume Analysis at Nanoscale Resolution. PLoS ONE 8(3): e59573. doi:10.1371/journal.pone.0059573
- [2] Powner M., Scott A., Zhu M., Munro P., et al. (2011): Basement membrane changes in capillaries of the ageing human retina. Br J Ophthalmol 2011;95:9 1316-1322 Published Online First: 23 May 2011
- [3] Maco B., Holtmaat A., Cantoni M., Kreshuk A., Straehle C. N., et al. (2013): Correlative In Vivo 2 Photon and Focused Ion Beam Scanning Electron Microscopy of Cortical Neurons. PLoS ONE 8(2): e57405. doi:10.1371/journal.pone.0057405

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1 Correlating Light Microscopy and Transmission Electron Microscopy

1.1 Fluorescent imaging followed by resin embedding and postlabeling

1.1.1 Sample: Perfused bird brain

Imaging: LSM → TEM

Differentially labeled distinct populations of projection neurons in the songbird brain. The sample was prepared for fluorescent LM and further processed for EM and additional fluorescent imaging afterwards.

Preparation of bird brain (Imaging: LSM, LM and TEM)

1 Labels	Lucifer yellow, Alexa Fluor® 647, Tetramethylrhodamine, rabbit anti-lucifer yellow antibody goat anti-rabbit Alexa Fluor® 594
Fluorescent Labeling	Fluorophores are injected into the nucleus of arcopallium, into Area X, and nucleus uveaformis respectively. In addition postembedding immunolabeling was performed on serial sections
2 Fixation	<ol style="list-style-type: none"> Perfusion with 20 µl heparin, 5 ml 0.9% NaCl 2% PFA, 0.075% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4), 20 min Remove brain and postfix in same fixative, 1 h 60 µm thick sagittal vibratome sections
3 LM Imaging	<ol style="list-style-type: none"> LSM imaging with 63x, NA 1.3 glycerol objective Stacks with 60 optical sections recorded
4 Postfixation	<ol style="list-style-type: none"> Wash the sections in Na-cacodylate buffer (0.1 M, pH 7.4) Fix with 1.5% Potassium ferrocyanide, 1% OsO₄ in Na-cacodylate buffer (40 min) 1% OsO₄, in Na-cacodylate buffer, 1 h 1% uranyl acetate, in distilled water, 1 h Dehydration Flat-embed sections in Durcupan ACM resin Cure for 48 h at 52 °C
5 Ultramicrotomy	<ol style="list-style-type: none"> After curing, HVC neurons were found again in the light microscope Resection and attachment to a blank resin block Serial sections with 60-90 nm thickness Collect sections on piloform coated nickel slot grids
6 Immunofluorescence	<ol style="list-style-type: none"> Treat grids with 1% periodic acid, 10 min Wash 15 times in distilled water Wash 2 times in TPBS pH 7.4, 10 min each Wash in TPBS, 5% goat serum, 30 min Wash in TPBS, 1% goat serum, 10 min Invert grids on droplets of diluted primary antibody, 1.5 h (antibody: 1:50, rabbit anti-Lucifer yellow)

-
- 6 Immunofluorescence**
- g. Wash 4 times with Tris-HCl (0.05 M, pH 7.4), 10 min
 - h. Invert grids on droplets of diluted secondary antibody, 1.5h (antibody: 1:50, Alexa Fluor® 594 goat anti-rabbit IgG)
 - i. Wash 15 times in distilled water
-
- 7 LM Imaging**
- a. Fluorescence light microscopy with 40x, NA 0.6 air
-
- 8 Additional Staining**
- a. Treat grids with 1% Uranyl acetate, 3 min
 - b. Wash 3 times in distilled water, 30 s each
 - c. Stain with Reynold's lead citrate, 2 min
 - d. Wash 3 times in distilled water, 30 s each
-
- 9 EM Imaging**
- a. TEM imaging
-

References:

Oberti D., Kirschmann M. A. and Hahnloser R. H. R.: Correlative microscopy of densely labeled projection neurons using neural tracers. *Frontiers in Neuroanatomy* 2010; 4:24.

1.1.2 Sample: Photoreceptor precursor cells from mice retina

Imaging: LSM → TEM

Neonatal double transgenic reporter mice in which the outer segments are selectively labeled with a green fluorescent protein while cell bodies are highlighted with a red fluorescent protein are investigated by CLEM. A series of CLEM and non-CLEM preparations were performed. Here only the CLEM protocol is replicated.

Mouse retina (Imaging: LSM, LM and TEM)

1 Labels	Rabbit-anti-GFP antibody, anti-rabbit AlexaFluor 488 antibody, Protein A 10 nm gold, DAPI
Fluorescent Labeling	Postembedding immunolabeling
2 Fixation	<ul style="list-style-type: none"> a. Retina pieces were fixed in 4% PFA in 0.1 M phosphate buffer, pH 7.4 overnight at 4 °C b. PLT (progressive lowering of the temperature) embedding in K4M resin <ul style="list-style-type: none"> i. Dehydration in a graded series of ethanol at progressively lower temp. ii. Infiltration in mixtures of ethanol/K4M at -35 °C iii. Polymerization with UV light at -35 °C
3 Ultramicrotomy	<ul style="list-style-type: none"> a. Cutting of ultrathin sections b. Place sections on 200 mesh nickel finder grids
4 Immunofluorescence	<ul style="list-style-type: none"> a. Blocking of sections with 1% BSA in PBS b. Stain with rabbit anti-GFP antibody and protein A gold c. Postfixation with 1% glutaraldehyde/PBS d. Incubation with anti-rabbit AlexaFluor 488 e. Counterstain with DAPI
5 LM Imaging	<ul style="list-style-type: none"> a. Mount grids between two coverslips in 50% glycerin/water b. Fluorescence microscopy c. De-mount grids and wash several times in water
6 2nd Staining	<ul style="list-style-type: none"> a. Stain with aqueous uranyl acetate
7 EM Imaging	<ul style="list-style-type: none"> a. Transfer to the EM for inspection

References:

Eberle D., Kurth K., Santos-Ferreira T., Wilson J., Corbeil D., Ader M.: Outer segment formation of transplanted photoreceptor precursor cells. PLoS ONE 7(9): e46305. doi:10.1371/journal.pone.0046305

1.1.3 Sample: Connexin trafficking, HeLa expressing Cx43

Imaging: LSM, multiphoton → TEM

Fluorescent labeling (in vivo) and photoconversion to generate high density precipitates at fluorescent sites visible in the EM to show connexin trafficking. A series of CLEM and non-CLEM preparations were performed. Here, only the CLEM protocol is replicated.

HeLa cells (Imaging: LSM, multiphoton, LM and TEM)

1 Labels	a. FIAsh-EDT ₂ b. ReAsH-EDT ₂
Fluorescent Labeling (in Vivo)	a. FIAsh or ReAsH is used at final concentrations of 1 and 2.5 μM in the presence of 10 μM EDT b. Incubate HeLa cells expressing Cx43-TC for 1 h at 37 °C in 1x HBSS supplemented with D+ glucose (1g/l) c. Non-bound and nonspecifically bound ligands are removed by washing with EDT (250 μM in HBSS+glucose) d. Incubate cells for 4 to 8 h in complete medium e. Second round of labeling to stain the newly synthesized proteins f. Ligand used in the second round are FIAsh for the initially ReAsH labelled cells and vice versa utilizing the same concentrations and incubation times as in the first round
2 LM Imaging	a. Live cell imaging in multiphoton mode b. Cells were kept in HBSS supplemented with glucose and 10 μM EDT
3 Fixation	a. Fix cells in 2% glutaraldehyde, Na-cacodylate buffer (0.1 M/pH 7.4) for 20 min after live cell imaging b. Rinse with buffer c. Treat with KCN (20 mM), aminotriazole (5 mM), glycine in buffer (50 mM) for 5 min, for non-specific background reduction
4 DAB Photoconversion	a. Add 1mg/ml diaminobenzidine in 0.1 M oxygenated sodium cacodylate to the culture dish b. Irradiate with 585 nm light from a Xenon lamp for 10 to 15 min until a brownish reaction product appears in place of a red fluorescence c. Wash cells with 1% osmium tetroxide for 30 min d. Rinse with distilled water
5 Resin Embedding	a. Dehydrate in ethanol b. Embed in Durcupan resin c. Polymerize at 60 °C, 48 h
6 EM Imaging	a. TEM imaging

References:

Gaietta G., Deerinck T. J., Adams S. R., Bouwer J., Tour O., Laird D. W., Sosinsky G. E., Tsien R. Y. and Ellisman M. H.: Multicolor and electron microscopic imaging of connexin trafficking. Science 2002 (296), 503-507

1.1.4 Sample: Human hepatoma cells

Imaging: LSM/Spinning disk → TEM

Protocol to use gridded coverslips and cryo-immunogold labeling for visualizing fluorescently tagged proteins in light and electron microscopy.

Human hepatoma cells (Imaging: live cell LM/LSM/Spinning disk and TEM)

1 Production of Gridded Coverslips	<ul style="list-style-type: none"> a. Clean gridded coverslips and glass slides with ethanol and water b. Glue gridded coverslip onto a glass slide with a paper sticker and wax c. Prepare a 1.1% formvar solution in chloroform d. Coat another glass slide with Formvar to create a Formvar film. e. Scrape the sides of the Formvar coated glass slides with a razor blade and float the Formvar film onto a water bath f. Place the glass slide with the gridded coverslip vertically on the edge of the film and lower it into the water (in this way you should get a wrinkle free Formvar film onto the gridded coverslip) g. Dry the Formvar film on the gridded coverslip overnight h. Place the glass slide with the gridded coverslip onto a heated stage, wait until the wax melts and detach the coverslip i. Clean the non-coated side with ethanol to remove remaining wax pieces
2 Gelatin Coat	<ul style="list-style-type: none"> a. Place the Formvar-coated side of the gridded coverslip on a droplet of 1% gelatin in distilled water b. Incubate 15 min at room temperature c. Immediately rinse 2x with distilled water d. Fix the gelatin coat onto the coverslip for 15 min with 1% glutaraldehyde e. Rinse with distilled water f. Dry overnight
3 Mounting into a Cell Culture Dish	<ul style="list-style-type: none"> a. Place a metal cylinder on a heated platform b. Position a cell culture dish containing a 14 mm hole upside down onto the heated metal cylinder c. Position the grid carefully – coated side upside down – over the cell culture dish hole d. Apply dots of melted wax to the edges of the coverslip, capillary forces will pull the wax between the coverslip and the dish e. Remove the dish from the heated block f. Once the wax is cooled it will glue the coverslip to the dish g. Sterilize the culture dish with the gridded coverslip under UV light for at least 30 min h. Plate cells onto the coated, gridded coverslip
4 Labeling	<ul style="list-style-type: none"> a. Depending on your cell culture and experiment you will have either cells stably expressing fluorescent constructs or you need to use fluorescent and/or immunogold probes
5 LM Imaging	<ul style="list-style-type: none"> a. Image the cells with your desired equipment b. Use DIC for mapping grid locations

6 Fixation	<p>a. Fix the cells with a mixture of 4% PFA, 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4)</p> <p>Note: conditions need to be optimized for the respective experiment!</p>
7 LM Imaging	<p>a. Record desired imaged data</p> <p>b. Use DIC for mapping grid locations</p>
8 Postfixation	<p>a. Clean immersion oil from the coverslip with ethanol</p> <p>b. Detach coverslip from cell culture dish</p> <p>c. Fix cells on the coverslip overnight in 2% PFA, 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C to optimize ultrastructural preservation</p>
9 Embedding for Immuno EM	<p>a. Mount the coverslip onto a glass slide with Mowiol, cells facing upside down</p> <p>b. Locate your region of interest using the DIC map, mark the region with a dot</p> <p>c. Remove the coverslip from the glass slide by immersing it into PBS</p> <p>d. Place the cells facing up into a petri dish</p> <p>e. Rinse with PBS containing 0.1% BSA</p> <p>f. Incubate with 800 µl of 6% gelatin in PBS at 37 °C for 30-60 min in a wet chamber to allow the gelatin to disperse in between the cells</p> <p>g. Rinse with PBS (containing 0.1% BSA)</p> <p>h. Add 400 µl 12% gelatin with erythrocytes at 37 °C, and allow it to solidify for 30 min at 4 °C.</p> <p>i. Fix the sample for 30 min in 0.5% PFA in PBS at 4 °C</p> <p>j. Rinse 2x with cold PBS</p> <p>k. Incubate for 30 min with cold 2.3 M sucrose then replace the solution with fresh sucrose and place the sample on a rocker for 3 days at 4 °C to allow infusion of the gelatin and cell layer</p>
10 Cryosections (*)	<p>a. Use a razor blade to cut out the marked area</p> <p>b. Mount the block on a pin with the erythrocyte layer facing the specimen holder</p> <p>c. Freeze in liquid nitrogen</p> <p>d. Cut (about 4-5) ~70 nm thick cryosections and collect them on carbon-coated Formvar grids (sectioning temperature was -100 °C!)</p>
11 Immunogold Labeling of Cryosections	<p>a. Place every 5th grid on 2% gelatin in PBS at 37 °C for at least 90 min to remove the fixed layer of gelatin</p> <p>b. Immunogold labeling according to (Slot et al. see below*) Antibody to mGFP marked with Protein A-10 nm gold was used</p>

12 Imaging and Relocation

- a. View the labeled sections in the EM
 - b. Use the previously taken DIC map to locate the position of the cell on the grid and the fluorescent z-dimension from the image stacks to determine which grid (and section) corresponds to the imaged plane
 - c. After matching further grids can be chosen for labeling
-

(*) Slot J. W. and Geuze H. J.: Cryosectioning and immunolabeling. *Nat. Protoc.* 2, 2480–2491 (2007).

References:

van Rijnsoever C., Oorschot V., Klumperman J.: Correlative light-electron microscopy (CLEM) combining live-cell imaging and immunolabeling of ultrathin cryosections. *Nat Methods*, 2008 (5) 973-980

1.1.5 Sample: HEK 293 A cell culture

Imaging: Widefield/LSM → TEM

HEK 293A cells stably transfected with GFP-LC3 were used to investigate autophagy. For relocation, gridded coverslips were used.

HEK293A stably transfected cells (Imaging: wide field, LSM and TEM)

1 Labels	HEK 293A cells stably transfected with GFP-LC3
Fluorescent Labeling	Fluorescently labeled fusion protein
2 Cell Culture	<ol style="list-style-type: none"> HEK 293A cells were cultured under sterile standard cell culture conditions HEK 293A cells are seeded onto 13 mm round gridded coverslips for easy relocation of cell positions. (CELLocate, Eppendorf, or Bellco Biotechnology) If cells detach easily you can pre-coat the coverslips with poly-D-lysine (1 mg/ml) for 5 min Followed by 3x washes in distilled water at room temperature
3 Experiments	<ol style="list-style-type: none"> Culture your cells to optimal confluence Perform your experiments as you are used to
4 Fixation	<ol style="list-style-type: none"> Wash the coverslips in PBS once Fix the cells for 30 min at room temperature in a mixture of 4% PFA, 2% glutaraldehyde, 0.1 M Na-cacodylate, pH 7.2; prewarmed to 37 °C Wash 3x with PBS Fixed cells remain in PBS for image acquisition
5 LM Imaging	<ol style="list-style-type: none"> Find regions of interest and record the positions with the help of the grid pattern in the coverslip Capture different contrasts (e.g. phase contrast and fluorescence) for easy recognition of defined regions (A 20x lens is a good working magnification for overview images that can be used for orientation) Capture high magnification images and Z-stacks using desired contrasts and settings. Proceed with processing the samples for EM
6 Postfixation and Resin Embedding	<ol style="list-style-type: none"> Remove all PBS Add 2% osmium tetroxide (OsO_4) in 0.1 M Na-cacodylate, pH 7.2 Incubate for 1 h on ice in the dark in the fume hood. OsO_4 will fix and stain the lipids in the cells Remove OsO_4 and wash the cells 2x with 0.1 M Na-cacodylate pH 7.2 Incubate cells in freshly made 1% tannic acid in 0.05 M Na-cacodylate, pH 7.2 for 40 min (this step improves the contrast) Wash 2x with 0.05 M Na-cacodylate, pH 7.2

6 Postfixation and Resin Embedding	<p>g. Wash 1x in double-distilled H₂O</p> <p>h. Dehydrate cells by incubating in 70%, then in 90% ETOH for a few minutes</p> <p>i. Incubate with 2x 100% ETOH for 10 min (store cells in 100% ETOH with desiccant)</p> <p>j. Mix a 1:1 solution of propylene oxide:EPON. (EPON is prepared from 20 ml Araldite cy212, 0.8 ml DMP-30 and 25 ml DDSA)</p> <p>k. Add this propylene:EPON mix to an aluminium dish (Note: propylene oxide will dissolve most laboratory plasticware)</p> <p>l. Remove coverslip from 100% ETOH and transfer it (cell facing up) into the aluminium dish and incubate the sample for 30 min</p> <p>m. Remove the old EPON mixture as much as possible and replace the old EPON mixture with fresh 100% EPON, incubate for 1 h</p> <p>n. Remove EPON and replace with fresh EPON, incubate for 1 h</p> <p>o. Mount the coverslip (cell side upside down) on the flat ends of prepolymerized EPON stubs made in cylindrical capsules. Pay attention to not have air bubbles between the stub and the coverslip.</p> <p>p. Gently remove excessive EPON from the sides of the coverslip</p> <p>q. Make sure that the area on the stub has the area of the grid in which the cells of interest are found</p> <p>r. Bake at 60 °C overnight to polymerize the EPON</p> <p>s. Remove the coverslip from the now embedded cells by dipping it in liquid nitrogen for a few seconds. Remove all remaining glass pieces.</p>
7 Ultramicrotomy	<p>a. Locate the cells in the grid region of interest using the eyepiece of the microtome</p> <p>b. Trim the block with a single sided razor to your region of interest</p> <p>c. Cut serial thin sections (~70 nm) for conventional TEM or thick sections (~250 nm) for electron tomography. Calculate the number of sections to reach the desired location in z from the section thickness and information from the Z-stacks acquired earlier on the light microscope</p> <p>d. Place the ribbon of sections on a Formvar coated slot grid</p>
8 EM Contrast Enhancement	<p>a. Stain sections with lead citrate (prepare lead citrate from 1.33 g lead nitrate, 1.76 g sodium citrate in 50 ml ddH₂O, shake for 1 min then intermittently for 30 min. Add 8.0 ml of fresh 1N NaOH)</p> <p>i. Place small drops (20 µl) on clean parafilm in a petri dish</p> <p>ii. Place 3-5 NaOH pellets into the petri dish (NaOH pellets absorb CO₂ and prevent precipitation of lead citrate with ambient CO₂ on the sections)</p> <p>iii. Invert the grids onto the lead citrate drops for 5-10 min</p>

9 EM Imaging

- a. Examine the section that was close to the coverslip first (it will have the highest similarity to the phase contrast images)
 - b. Once the region is located acquire an overview mosaic image at low magnification for relocating and comparison with the LM images
 - c. Acquire high resolution images.
-

References:

Razi M., Tooze S. A.: Correlative light and electron microscopy. *Methods Enzymol*, 2009 (452), 261-275

1.2 Protocols for preserving fluorescence in resin embedded samples

1.2.1 Sample: Native human skin

Imaging: LSM → TEM

Modified freeze substitution protocol to introduce fluorescent dyes during freeze substitution. Fluorescent labelling is performed after resin embedding on plastic sections. LSM was performed on the resin embedded sample. In addition the authors tested a number of different dyes. Their table is replicated after the protocol.

Human native skin biopsies (Imaging: LSM → TEM)

1 Labels	Various dyes introduced during freeze substitution (see table p. 17)
Fluorescent Labeling	Fluorescent dyes were added to the substitution medium prior to cooling
2 Sample Fixation	<ol style="list-style-type: none"> a. Take human skin samples with standard 2 mm punch biopsies b. Uncover the dermis by careful removal of underlying adipose tissue c. Cut the sample into smaller pieces (500 – 2000 µm diameter, 200 µm thick) d. Place the cut samples into aluminium planchettes filled with 1-hexadecene and transfer the samples into a high-pressure freezer (e.g. HPM 010) e. After freezing, the samples are stored in liquid nitrogen
3 Freeze Substitution	<ol style="list-style-type: none"> a. Perform a freeze substitution in an automated freeze substitution (AFS) unit for 64 h (40 h at -90 °C, 12 h at -70 °C, 12 h at -50 °C) b. Substitution medium is acetone saturated with uranyl acetate. The fluorescent dyes are directly added to the substitution medium prior to cooling c. Before starting the AFS, remove 1-hexadecene carefully under liquid nitrogen with a precooled small brush. d. Transfer the sample into transfer vessels (Hohenberg et al. 1994) e. Place them in 1.5 ml Eppendorf tubes filled with substitution medium (during AFS the dyes will co-penetrate into the sample, uranyl acetate serves as a fixative and heavy metal stain) f. After AFS, wash the samples with acetone for 2 h at -50 °C) g. Wash 2x with ETOH for 1 h at -50 °C each
4 Resin Embedding	<ol style="list-style-type: none"> a. Samples are either infiltrated with <ol style="list-style-type: none"> i. EPON 812 in increasing concentrations in ETOH (33% v/v for 2 h at -50 °C, 67% v/v for 2h at 4 °C, and 100% twice overnight and 2 h at room temperature. Polymerize in fresh pure EPON for 72 h at 60 °C ii. HM20 in ETOH (33% v/v for 2 h at -50 °C, 67% v/v for 2 h at -50 °C, and twice 100%, overnight and for 2 h at -50 °C). UV-polymerization was carried out in fresh pure HM20 for at least 4 days at -50 °C.
5 Preparation for LM Imaging	<ol style="list-style-type: none"> a. Remove the surrounding resin b. Produce a plain sample blockface with a diamond trimming knife in an ultramicrotome (Don't cut into the sample yet) c. Mark the resin block with a 21-gauge needle (resulting in a pyramidal 3D marker in the resin block)

6 LM Imaging	a. Image with an LSM equipped with an Ar/Kr laser and oil immersion objectives (20×/NA 0.7, 40×/NA 1.0, 63×/NA 1.3, 100×/NA 1.3) b. The depth of the located areas of interest is estimated with the LSM software measurement tools.
7 Ultramicrotomy	a. Serial thin sections are produced according the depth measurements from the LSM. The depth is measured from the number of sections and the section thickness
8 Poststaining	a. Poststain the sections with uranyl acetate and lead citrate
9 EM Imaging	a. Examine sections in the TEM and assessing different sections to LM data

Analysis of different dyes for Freeze substitution

Dye	Highlighting	Image quality
1.8 ANS	collagen and/or elastic fibres	Fair
Acridine	orange nuclei, cytoplasm	Good
Bodipy 560	nuclei, cell borders, stratum corneum; whole dermis	Fair
DCVJ	nuclei; collagen and/or elastic fibres	Fair
DiIC18	lipophilic domains, cell membranes	Good
DiOC6	cytoplasm (lipophilic domains), stratum corneum	Good
Nile Blue	sulphate nuclei, stratum corneum; collagen and/or elastic fibres	Good
Nile Red	nuclei, cytoplasm, stratum corneum	Poor
Nonyl Acridine Orange	nuclei, cytoplasm, stratum corneum	Good
Oregon Green	nuclei; collagen and/or elastic fibres	Fair
Safranin T	nuclei, cytoplasm, stratum corneum; collagen and elastic fibres	Good
Sudan III	nuclei; whole dermis	Fair
Tannin	nuclei, stratum corneum	Poor
Uranyl Acetate	nuclei, stratum corneum	Poor

Note: this assessment is valid only for the Ar/Kr laser; staining and contrast with the respective dyes may be different for other excitation wavelengths. (table reproduced from S. S. Biel et al. J. Microsc, 212 (1) p. 96)

References:

Biel S. S., Kawaschinski K., Wittner K.-P., Hintze U. and Wepf R.: From tissue to cellular ultrastructure: closing the gap between micro- and nanostructural imaging, J. Microsc, 2003, 212 (1) 91–99

1.2.2 Sample: Punch biopsy of human axillary skin, sweat glands

Imaging: Widefield/LSM → TEM

Axillary skin samples were obtained through punch biopsies, chemically fixed or high pressure frozen. Either frozen or resin embedded sections were used. Fluorescent dyes were added during freeze substitution. Here the focus was on samples sizes too large to use in high pressure freezing. In addition to introducing fluorescent dyes during freeze substitution the resin embedded sections were immunolabelled.

Human native skin biopsies (Imaging: LSM → TEM)

1 Labels	Various dyes introduced during freeze substitution (see table on page 17)
Fluorescent Labeling	Fluorescent dyes were added to the substitution medium prior to cooling
2 Sample Preparation	a. Take standard punch biopsies (4 mm) of human axillary skin
3 Sample Fixation	<p>a. Samples are either fixed by chemical or physical fixation</p> <p>b. Chemical fixation with either Karnovsky or IEM:</p> <ol style="list-style-type: none"> i. Karnovsky: 0.2% formalin, 1.25% glutaraldehyde in 0.2 M Na-cacodylate buffer, PLP [0.01 M sodiumperiodate, 0.075 M L-Lysine, 2% paraformaldehyde in phosphate-buffered saline (PBS) ii. IEM: 4% paraformaldehyde, 0.01% glutaraldehyde in 0.05 M HEPES buffer iii. In either case fix for 24 h at 4 °C iv. Place sample in cryoprotectants for another 24 h at 4 °C (Cryoprotectants can be saccharose, glycerine, DMSO or a mixture (1:1) of glycerine and DMSO; each in 30% water) v. It is possible to add 1% uranyl acetate, 1% OsO₄ to the cryoprotectants vi. Quarter biopsies for freeze substitution to enhance mass transfer vii. Biopsies for cryosectioning can remain as they are viii. Wash samples and plunge into liquid ethane ix. Store in liquid nitrogen until further processing <p>c. Physical fixation</p> <ol style="list-style-type: none"> i. High pressure freezing of the samples
4 Sample Embedding	<p>a. Samples are either embedded at room temperature with freeze substitution or kept for cryo-sectioning (see step 5)</p> <p>b. EPON embedding at room temperature</p> <ol style="list-style-type: none"> i. Wash samples twice with Na-cacodylate buffer ii. Stain overnight with fluorescent dyes (table p.17) iii. Wash again with Na-cacodylate buffer iv. Dehydrate samples with different concentrations of ETOH v. Infiltrate with increasing concentrations of EPON (33% (v/v) EPON in ethanol (2 h), 67% (v/v) in ethanol (2 h) and 100% (twice, overnight and 2 h) vi. Polymerize in fresh, pure EPON, 72 h at 60 °C <p>c. Freeze substitution</p> <ol style="list-style-type: none"> i. Perform freeze-substitution for 64 h (40 h at -90 °C, 12 h at -70 °C, 12 h at -50 °C) in acetone with saturated uranyl acetate as substitution medium. Fluorescent dyes are added to the substitution medium prior to cooling (table p.17) ii. After substitution, wash the sample 2x with ETOH, 1 h at -50 °C each iii. Infiltrate samples with increasing concentrations of HM20 in ETOH, (33% (v/v), 2 h at -35 °C, 67% (v/v), 2 h at -35 °C and 100%, overnight and again 2 h at -35 °C iv. UV polymerize the sample in fresh pure HM20 for 72 h at -35 °C

-
- 5 Sample Preparation**
- a. Frozen samples are used to produce cryosections
 - i. Cut frozen samples into 8 μm thick sections at $-25\text{ }^{\circ}\text{C}$ in a cryostat
 - ii. Place the sections on glass slides and examine the sections with a standard LM
 - b. Embedded samples are processed as follows:
 - i. Remove the surrounding resin
 - ii. Produce a plain blockface in the ultramicrotome using a trimming knife
 - iii. Don't cut into the sample yet
-
- 6 LM Imaging**
- a. Examine the blockface with LSM using a Ar/Kr laser and
 - b. Oil different objectives 10x/NA 0.4 (dry), 20x/NA 0.7 (oil immersion), 40x/NA 1.0 (oil immersion)
 - c. Map the depth of the desired areas of interest and measure the z-position with the LSM software
-
- 7 Ultramicrotomy**
- a. Produce semi-, or ultrathin sections in an ultramicrotome with a diamond knife
 - b. Use the stepsize and section thickness to calculate the depth
-
- 8 Postprocessing**
- a. For EM, the sections are stained with uranyl acetate and lead citrate
-
- 9 EM Imaging**
- a. Acquire high resolution EM images
 - b. Assign EM images to the respective LM data
-
- 10 Immunolabeling**
- a. In addition immunolabeling is performed on either cryosections or semi-thin sections (200 nm) placed on masked glass coverslips
 - b. Cryosections/semithin sections:
 - i. Treat sections with 10% normal donkey serum, 2 h
 - ii. Wash 2x (in 1% fish gelatin and 0.8% BSA in PBS)
 - iii. Incubate with mouse anti-milk fat globule anti-serum, 1 h
 - iv. Wash 2x (in 1% fish gelatin and 0.8% BSA in PBS)
 - v. Incubate with Cy 2 conjugated donkey anti-mouse IgG (1:200), 1 h
 - c. Ultrathin (70 nm) sections:
 - i. Treat ultrathin sections as above
 - ii. Use Protein A bound to 10 nm gold as secondary antibody
-

Note: According to the authors; for the sample used in this study:

- Chemical fixation in IEM buffer was optimal for preservation of antigenicity and structure.
 - Chemical fixation in Karnovsky's resulted in good structure preservation but impaired antigenicity.
 - Utilizing 30% saccharose as cryoprotectant and 1% uranyl acetate provided the best structural preservation without impairing antigenicity.
 - Serial cryosectioning was the method of choice to estimate to whole dimensions of the sample and were used successfully for 3D reconstruction.
-

References:

Wilke K., Wick K., Keil F. J., Wittern K.-P., Wepf R., Biel S. S.: A strategy for correlative microscopy of large skin samples: towards a holistic view of axillary skin complexity. *Exp Dermatol* 2008 (17), 73-81

1.2.3 Sample: Labeling of Ultrathin Resin Sections

Imaging: Widefield → TEM

Protocols for fluorescent and gold labeling on the same ultrathin sections of methacrylate (Lowicryl K4M) embedded samples of mice retina and in the Microtubular Manchette of mouse spermatids.

Mouse Retina/Mouse Spermatids (Imaging: widefield → TEM)

1 Labeling	Protein A-10 nm gold, IgG gold, Alexa Fluor® 488, Alexa Fluor® 555 and alternatively silver-enhanced FluoroNanogold as correlative marker
Fluorescent Labeling	Labeling was done on embedded and sectioned samples.
2 Sample Fixation	<ul style="list-style-type: none"> a. Samples are fixed in mild fixatives to preserve antigenicity b. Fix in 2-4% PFA with or without low concentrations of glutaraldehyde (0.05-0.5%) c. Mouse retina samples are fixed in 4% PFA in 0.1 M phosphate buffer, pH 7.4 d. Mouse testis samples are fixed after dissection in 4% PFA, 0.1 M phosphate buffer, pH 7.4 e. Alternatively, samples can be fixed by high pressure freezing but large tissue blocks have to be dissected further before freezing
3 Dehydration	<ul style="list-style-type: none"> a. Dehydrate samples in a series of ETOH/water mixtures at progressively lower temperature (PLT) <ul style="list-style-type: none"> i. 30%, 50% at 0-4 °C, 45 min, in the fridge or on ice ii. 70% (and optional 80%) at -20 °C, 1 h each, in the freezer iii. 90%, 96%, 2x 100% at -35 °C, 1 h each in an automated freeze substitution device
4 Infiltration	<ul style="list-style-type: none"> a. Infiltrate the sample at -35 °C in an automated freeze substitution device <ul style="list-style-type: none"> i. ETOH:K4M (1:1), 1 h ii. ETOH:K4M (1:2), 1 h iii. Pure K4M overnight iv. Pure K4M 1-2x, 3 h
5 Embedding	<ul style="list-style-type: none"> a. Embed samples in either 0.5 ml reaction tubes or in flat embedding forms b. Polymerize K4M with UV light for 24-48 h at -35 °C c. If 0.5 ml reaction tubes are used cover tube lids with strips of aluminum foil to trigger resin polymerization by indirect UV radiation d. Remove strips after 12-24 h and irradiate the remaining 12-24 h without strips. e. Transfer blocks to the fume hood and let unpolymerized K4M evaporate f. The sample is ready when the K4M "smell" is gone (Note: this is mentioned in the original publication and probably refers to the faint resin smell that is present outside the fume hood. Please be aware that resin fumes are highly toxic and should only be handled in fume hoods and never be inhaled.)
6 Ultramicrotomy	<ul style="list-style-type: none"> a. Cut semi-thin sections b. Stain with toluidine blue/borax for easy selection of potential interesting regions in the light microscope c. For correlative immunolabeling collect sections preferably on finder grids (but slot or mesh grids can also be used)

-
- 7 Correlative Immunolabeling/Fluorescent Labeling**
- a. A general strategy is to label primary antibodies with protein A-gold or IgG gold and fill up unoccupied binding sites with secondary antibodies conjugated to Alexa Fluor® 488 or Alexa Fluor® 555. All steps are carried out at room temperature
 - b. Block the sample 2x for 5 min in 1% BSA fraction V, in PBS
 - c. Incubate the sample with the primary antibody (rabbit anti-GFP in 1% BSA/PBS, 1h)
 - d. Wash 4-5x in PBS, 2 min each
 - e. Optional:
 - i. Bridging antibody: e.g. rabbit anti-mouse IgG (necessary if primary antibody is a mouse monoclonal and Protein A is used)
 - ii. FluoroNanogold-conjugated antibody
 - iii. After bridging antibody or FluoroNanogold: Wash 5x with PBS, 2 min each
 - iv. Proceed with step "m – DAPI stain"
 - f. Protein A gold or IgG gold in 1% BSA/PBS, 30 min to 1h
 - g. Brief washes (3x in PBS)
 - h. Wash 4x in PBS, 2 min each
 - i. 1% glutaraldehyde in PBS, 5 min (in the fume hood), this will fix the Protein A gold and can be omitted when IgG gold is used (this is a postfixation step; it is optional and depends on how much time the imaging of specific fluorescence will take)
 - j. Wash 4-5x in PBS, 2min each
 - k. Incubate with secondary antibody conjugated to Alexa Fluor® 488 or Alexa Fluor® 555 in 1% BSA/PBS
 - l. Wash 4-5x in PBS, 2 min each
 - m. Stain with 1 µg/ml DAPI, 5-10 min
 - n. Wash 5x in water, 1 min each
-
- 8 LM Imaging**
- a. Mount the grids with the fluorescently stained samples in 50% glycerin/water between a microscope slide and coverslip
 - b. Collect desired image data
 - c. Wash grid 5x in water, 1 min each
 - d. Optional if FluoroNanogold was used:
 - i. Additional: wash 5x in water, 1 min each
 - ii. Silver enhancement using the R-Gent SE-Silver enhancement kit, incubation 30-60 min
-
- 9 Poststaining**
- a. Stain with 2-4% uranyl acetate in water, 5-10 min
 - b. Wash 3x in water, 1 min each
-
- 10 EM Imaging**
- a. Collect high resolution TEM images of the sections
-

Different antibody combinations used in the study

Primary Antibody	IgG gold, bridging Ab, Prot A gold, FluoroNanogold	Prot A gold	Fluorescent marker	Silver enhancement (SE)
Anti-GFP (R-IgG)	Protein A gold	Protein A gold	G-anti-R Alexa Fluor® 488or Alexa Fluor® 555	
Anti- α -Tubulin (M-Monoclonal)	Bridging Ab (R-anti-M IgG)	Protein A gold	G-anti-R Alexa Fluor® 488	
Anti- α -Tubulin (M-Monoclonal)	Bridging Ab (R-anti-M IgG)		G-anti-M Alexa Fluor® 488	
Anti- α -Tubulin (M-Monoclonal)	G-anti-M 10 nm gold		G-anti-M Alexa Fluor® 488	
Anti- α -Tubulin (M-Monoclonal)	G-anti-M 10 nm gold		D-anti-G Alexa Fluor® 488	
Anti- α -Tubulin (M-Monoclonal)	G-anti-M Alexa Fluor® 488 FluoroNanogold F(a,b) fragments			SE

Table reproduced from Fabig, G. et al. Methods in Cell Biology 111 (2012), 75-95. The original table references the figures in the publication also. Please refer to the original publication for the images.

References:

Fabig G., Kretschmar S., Weiche S., Eberle D., Ader M.* and Kurth T.: Labeling of Ultrathin Resin Sections for Correlative Light and Electron Microscopy, in: Methods in Cell Biology 111 (2012), 75-95.

1.2.4 Sample: Yeast and MDCK cells, HIV**Imaging: Widefield → TEM**

Mapping of fluorescent signals with high precision (<100 nm) for electron tomographic reconstructions to identify HIV particles and microtubule ends. Samples are *S. pombe* strains expressing RFP-mal3p and GFP-atb2p and *S. cerevisiae* strains expressing EGFP- and mCherry-tagged endocytic proteins and MDCK-H2B-RFP cells.

***S. pombe* and *S. cerevisiae* and MDCK strains (Imaging: widefield → TEM)**

1 Labels	GFP, RFP, EGFP and mCherry
Fluorescent Labeling	Cell strains stably expressing the fluorescent fusion proteins
2 HIV Infection and Fixation	<ol style="list-style-type: none"> Allow purified HIV-eGFP-delEnv particles to bind to the cell surface, 30–60 min on ice Cryo-immobilization by high pressure freezing
3 Freeze Substitution and Embedding	<ol style="list-style-type: none"> Process samples further by automated freeze substitution and embedding in an automated freeze substitution (AFS) unit Perform AFS at -90 °C for 48–58 h with 0.1% (w/v) uranyl acetate in glass distilled acetone. Addition of 1–3% water is desired in some cases to improve membrane contrast; this did not influence fluorescence. Raise temperature to -45 °C (5 °C/h) Wash samples in acetone Infiltrate with increasing concentrations (10, 25, 50, and 75%; 4 h each) of Lowicryl in acetone while the temperature is further raised to -25 °C Exchange 100% Lowicryl three times in 10 h steps UV polymerize at -25 °C for 48 h Raise temperature to 20 °C (5 °C/h) Continue UV polymerization for 48 h
4 Ultramicrotomy	<ol style="list-style-type: none"> Cut 300 nm sections with a diamond knife Place sections on a carbon coated 200 mesh copper grid (finder grids are optimal for relocating grid position later)
5 Correlative Labels	<ol style="list-style-type: none"> Pretreat FluoSpheres (0.02 µm, 365 nm/415 nm) with 0.1 % Tween 20, 10 min (to reduce intensity) Wash 2x, 10 min by ultracentrifugation at 100,000 g Resuspend in PBS Adsorb to the EM grids by placing grid face-down into 15 µl drops of FluoSpheres for 10 min Wash grid with 3 drops of water Blot dry with filter paper

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- | | |
|-----------------------|---|
| 6 LM Imaging | <ul style="list-style-type: none">a. Place grid in a 25-30 μl drop of water on a coverslipb. Close the sandwich with a second coverslip that has some vacuum grease along the rimc. During imaging the grid side with the section faces the objectived. During imaging record the positions on the grid (i.e. relative to identifiable structures etc.)e. Record images with desired settings and include the FluoSpheresf. Images at different focal planes might be necessary if the grid is not flat |
| 7 Poststaining | <ul style="list-style-type: none">a. Yeast sections are post stained with lead citrateb. MDCK sections are not post stainedc. Protein A-15 nm gold, is adsorbed on both sides of the grids |
| 8 EM Imaging | <ul style="list-style-type: none">a. EM tomography is performed in a high-tilt dual-axis holder in a $\pm 60^\circ$ tilt rangeb. Lower magnification tomograms at 2-3$^\circ$ increments and 2.5-5 nm pixel sizes are acquired for FluoSphere based-correlation |
-

Note: In this study high precision fiducial based correlation was carried out with a custom developed MATLAB software. Please refer to the publication for more information.

References:

Kukulski W., Schorb M., Welsch S., Picco A., Kaksonen M. and Briggs J. A .G.: Correlated fluorescence and 3D electron microscopy with high sensitivity and spatial precision. (2011) J. Cell Biol. Vol. 192 No. 1 111–119

1.2.5 Sample: THP-1 human monocytes, HUVEC, MDCK

Imaging: Integrated LSM/TEM

Two high-speed fixation protocols are demonstrated on three cell lines, THP-1 monocytes, human umbilical vein endothelial cells (HUVECs), and Madin-Darby canine kidney cells (MDCK-II). The two methods VIS2FIX_H and VIS2FIX_{FS} use either hydrated fixatives or freeze substitution methods

HUVEC, MDCK cells and THP-1 monocytes (Imaging: iLEM → TEM)

1 Labels	Alexa Fluor® 488
Fluorescent Labeling	Immunolabeling
2 Fixation and Embedding	<ul style="list-style-type: none"> a. Two different methods VIS2FIX_{FS} and VIS2FIX_H can be used b. High pressure freeze the samples c. Cut vitrified sections (similar to CEMOVIS) with 60-80 nm thickness at -150 °C in a cryo ultramicrotome d. Follow the VIS2FIX_{FS} or VIS2FIX_H protocol
3a VIS2FIX _{FS}	<ul style="list-style-type: none"> a. Place a trimmed Leica flow through-ring (6 mm height) in a reagent bath (~1 cm height) in an automated freeze substitution (AFS) unit. The reagent bath sits on top of three cold rings b. The AFS unit is cooled down to -90 °C c. Add 3 ml of precooled (-90 °C) acetone and fixatives (0.1% w/v uranyl acetate, 0.1-0.5% v/v glutaraldehyde, 0.2-0.5% OsO₄) into the flow-throughring d. Grids with vitrified sections are transferred into the AFS unit's chamber e. Use precooled tweezers to transfer the grid carefully (section upside down) into the fixative into an individual compartment of the flow through ring f. Start the AFS program as soon as the last grid is placed into the flow-through ring. <ul style="list-style-type: none"> i. AFS program: ii. -90 °C, 15 min iii. Increase to -60 °C, 13 min (maximum slope 138 °C) iv. -60 °C, 15 min v. Increase to -20 °C, 18 min (maximum slope 133 °C) vi. -20 °C, 15 min vii. Increase to 0 °C, 9 min (maximum slope 133 °C) g. If desired the fixative composition can be changed at any point during AFS h. Changing the fixative from -90 to -60 °C to 0.1% w/v uranyl acetate, 0.2% w/v OsO₄ in acetone yields good morphology for the used cell types i. The fixative is changed subsequently to 0.1% w/v uranyl acetate, 0.2% v/v glutaraldehyde in acetone j. At 0 °C wash the grids 5x in 0.2% v/v glutaraldehyde in acetone (~3 ml per wash step) k. Rehydrate the sections in a decreasing concentration of acetone; 0.2% v/v glutaraldehyde in 95%, 90%, 80%, 70%, 50%, 30%, 10% acetone in filtered (0.22 µm filter) water. l. Wash flow-through rings 3x with water

3a VIS2FIX_{FS}	<p>m. Remove grids from flow-through ring</p> <p>n. Dry back of the grids with slightly moist filter paper</p> <p>o. Wash grids 7x by floating them on drops of filtered water, 1 min each</p> <p>p. Use for immunolabeling or correlative labeling or store until processing</p>
3b VIS2FIX_H	<p>a. Prepare 2 ml fixative: 0.01-0.5% w/v OsO₄, 0.2% w/v uranyl acetate, 0.01-0.2% v/v glutaraldehyde in 0.1 M PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 6.9) on ice</p> <p>b. Place a final volume of 800 µl into the reagent bath</p> <p>c. Place the bath in an AFS unit on 3 cold rings in a tin and set it to -90 °C</p> <p>d. Cover the reagent bath with an aclar foil or petri dish lid, the fixative will freeze</p> <p>e. Transfer grids with the vitrified sections into the AFS</p> <p>f. Use precooled tweezers to place the grid with the sections upside down onto the frozen fixative</p> <p>g. Place the reagent bath with the grids into a cold petri dish to protect the bath from humidity</p> <p>h. Transfer everything onto a 40 °C hot plate</p> <p>i. Wait 4-5 min until the surface of the fixative is partially liquid and as soon as all the grids are floating place everything on ice</p> <p>j. Protect the reagent bath from light to limit oxidation of the OsO₄</p> <p>k. Fix for another 10 min</p> <p>l. Remove the grids from the fixative</p> <p>m. Wash 10x, 1 min each on drops of 0.1 PHEM buffer or filtered water</p> <p>n. Use for immunolabeling or correlative labeling or store until further processing</p>
4a Immunolabeling	<p>a. Quench free aldehyde groups by washing 5x for 2 min in drops of 0.1 M PHEM buffer containing 0.02 M Glycine</p> <p>b. Incubate with 1% w/v BSA in 0.1 M PHEM buffer, 15 min</p> <p>c. Incubate with primary antibody, 1 h (antibodies in blocking buffer were mouse monoclonal against PDI, 1:100, rabbit polyclonal against Caveolin, 1:100, rat monoclonal against Forssman glycolipid, 1:10)</p> <p>d. Wash 5x, 0.1% w/v BSA, 0.1 M PHEM, 2 min each</p> <p>e. Incubate sections labeled for PDI with an additional bridging antibody, rabbit anti-mouse Ig, 1:300 in blocking buffer.</p> <p>f. Wash 5x, 0.1% w/v BSA, 0.1 M PHEM, 2 min each</p> <p>g. Label sections with Protein A-gold, 20 min (1:80, in blocking buffer, dilution is batch dependent)</p> <p>h. Wash briefly 3x</p> <p>i. Wash 7x in 0.1 M PHEM buffer, 2 min each</p> <p>j. Incubate with 1% glutaraldehyde in 0.1 M PHEM buffer</p> <p>k. Wash 10x on drops of filtered water, 1 min each</p> <p>l. Stain for 5 min with 2% w/v uranyl oxalate in filtered water, pH 7</p> <p>m. Wash briefly in filtered water</p> <p>n. Embed in 0.4% w/v uranyl acetate in 1.8 w/v methyl cellulose on ice</p>

4b Correlative Labeling

- a. The protocol starts similar to the immunolabeling in 4a
- b. Primary antibody mouse monoclonal against LAMP-2 (1:150) is used
- c. Wash 5x, 0.1% w/v BSA, 0.1 M PHEM, 2 min each
- d. Incubate with rabbit anti-mouse and Protein A gold
- e. Wash 5x, 0.1% w/v BSA, 0.1 M PHEM, 2 min each
- f. Incubate with goat anti-rabbit Alexa Fluor® 488 (1:200)
- g. Wash 5x, 0.1 M PHEM, 2 min each
- h. Fix for 15 min in 4% w/v PFA
- i. Wash 10x on filtered water drops, 1 min each
- j. Stain 5 min with 2% w/v uranyl oxalate
- k. Wash briefly on filtered water
- l. Stain 5 min with 2% w/v uranyl acetate
- m. Embed in 1.8% w/v methyl cellulose on ice

5 LM/TEM Imaging

- a. Integrated LM/TEM or successively in the LM then TEM
-

References:

Karreman M. A, van Donselaar E. G., Gerritsen H. C., Verrips C. T. and Verkleij A. J., VIS2FIX: A High-Speed Fixation Method for Immuno-Electron Microscopy., Traffic 2011(12), 806-814

1.2.6 Sample: HUVEC, MDCK, C2C12, THP-1, punch biopsies

Imaging: Integrated LM/TEM

Comparison of the fluorescence intensity of six fluorescent probes in a dry, oxygen free environment relative to their performance in water. Also a freeze-substitution and a resin embedding protocol is described that yields excellent membrane contrast in the TEM but prevents quenching of the fluorescent immunolabeling.

MDCK, C₂C₁₂, THP-1, HUVEC, human and mouse biopsies (Imaging: LM → TEM)

1 Labels	Alexa Fluor® 488, Alexa Fluor® 532, Cy2, Cy3, FITC, TRITC
Fluorescent Labeling	Immunolabeling
2 Fixation	<ol style="list-style-type: none"> a. Fixation in 4% PFA, 12% gelatin cryoprotected in 1.8 M sucrose in water, overnight (described previously in Van Donselaar, E., et al. 2007, Traffic 8, 471–485) b. Plunge freeze sample blocks in liquid nitrogen
3 Freeze Substitution	<ol style="list-style-type: none"> a. Perform freeze substitution in 3 steps <ol style="list-style-type: none"> i. -90 °C, ≥ 48 h to fully replace water with methanol ii. Raise temperature to -60 °C (slope 2 °C/h) iii. -60 °C for 8 h iv. Raise temperature to -40 °C (slope 2 °C/h) v. -40 °C for 8h b. Alternatively use the following freeze substitution program <ol style="list-style-type: none"> i. -90 °C, 48 h ii. Raise temperature to -70 °C (slope 2 °C/h) iii. -70 °C for 12 h iv. Raise temperature to -50 °C (slope 2 °C/h) v. -50 °C for 12h vi. Perform Lowicryl infiltration and polymerization at -50 °C. UV polymerization is running for 120 h
4 Infiltration	<ol style="list-style-type: none"> a. Stepwise infiltration with Lowicryl HM20 (described previously in Schwarz, H., Humbel, B.M., 1989, Scanning Microsc. 3, 57–64) at -40 °C b. Perform UV polymerization for 48 h at -40 °C c. Continue UV polymerization for 24 h at room temperature
5 Ultramicrotomy	<ol style="list-style-type: none"> a. Cut thin sections b. Collect sections on grid
6 Immunolabeling	<ol style="list-style-type: none"> a. Perform immunolabeling according to Schwarz, H., Humbel, B.M., 1989, Scanning Microsc. 3, 57–64 b. Primary antibodies: rabbit anti-Caveolin, mouse anti-protein disulfide isomerase (PDI) c. Secondary antibodies are conjugated to the fluorophore as stated above (see table p. 22) Normalized fluorescent intensity of fluorophores in a dry and wet environment for the results on dry and wet fluorescence of these fluorophores)

-
- 7 LM/TEM Imaging**
- a. Image grids in an LM/TEM
 - b. Alternatively image grids at the LM, record finder grid locations for later correlation at the TEM
-

Fluorophore	Normalized fluorescence intensity in	
	Water	Dry (N ₂)
Alexa Fluor® 488	1.0 ± 0.42	0.52** ± 0.23
Alexa Fluor® 532	1.0 ± 0.3	0.58** ± 0.20
Cy2	1.0 ± 0.19	2.2** ± 0.49
Cy3	1.0 ± 0.23	0.9 ± 0.18
FITC	1.0 ± 0.23	1.1 ± 0.29
TRITC	1.0 ± 0.28	1.46** ± 0.40

Values reproduced from Karreman et al. J Struct Biol, 2012 (180), 382-386

Statistically relevant differences in fluorescence intensity of the same fluorophore are marked with asterisks (*, T-test). Two asterisks indicate a p value below 0.01.

References:

Karreman M. A., Agronskaia A. V., van Donselaar E. G., Vocking K., Fereidouni F., Humbel B. M., Verrips C. T., Verkleij A. J. and Gerritsen H. C.: Optimizing immuno-labeling for correlative fluorescence and electron microscopy on a single specimen. J Struct Biol, 2012 (180), 382-386

1.2.7 Sample: Tobacco plant (*Nicotiana tabacum*) and Arabidopsis plant (*A. thaliana*)

Imaging: LSM, 3D-SIM → TEM

Fluorescent preservation in plant tissue after resin embedding. Low temperature was used during fixation. In addition, different resins (LR White, methacrylate, Durcupan) were used. LR White embedding gave the best results.

Tobacco (*Nicotiana tabacum*) and Arabidopsis (*A. thaliana*) (Imaging: LSM, 3D-SIM → TEM)

1 Labels	GFP, YFP, RFP, Alexa Fluor® 594
Fluorescent Labeling	Plants expressing GFP, YFP, RFP; immunolabeling with Alexa Fluor® 594
2 Fixation/Embedding	<p>a. Tobacco grown from seeds, processed between 30 and 55 days old:</p> <ol style="list-style-type: none"> i. Cut the petiole and immediately submerge in 4% (w/v) PFA, 2% (w/v) glutaraldehyde, 50 mM PIPES, 1 mM CaCl₂ ii. Trim further under fixative to eliminate airlocks iii. Incubate under fixative for 60 min at room temperature to allow petioles to transpire the fixative via the xylem iv. Section transversely into 2 mm slices with a razor blade v. Return slices into fixative on a rolling bed, incubate 16 h at 8 °C in the dark vi. Wash 3x in 50 mM PIPES, 1 mM CaCl₂, 10 min each at 8 °C vii. Dehydrate in a graded series of ETOH (50%, 70%, 90% (v/v), containing 1 mM DTT) each step 2x, 15 min at 8 °C viii. Infiltrate with LR White diluted in 90% ETOH in different ratios (1:1, 1:2 and 1:3), supplemented with 1 mM DTT, 45 min each, at 8 °C ix. Follow with 2x changes of 100% LR White, 60 min, at 8 °C, final change is done at room temperature x. Polymerize in gelatin capsules at 50 °C, 24 h <p>b. Arabidopsis, processed 3 to 5 days after germination</p> <ol style="list-style-type: none"> i. Process the intact seedlings ii. Embed as described above iii. Fix seedlings expressing RFP with 1% glutaraldehyde
3 En Bloc Staining	<p>a. Add a drop of each dye, (1 µg/ml propidium iodide, 10 µg/ml calcofluor white) to the trimmed block face</p> <p>b. Allow to penetrate into the tissue, 20 min</p> <p>c. Rinse remaining dye from block face by immersing it in distilled water, 2 min</p>
4 Immunolocalization	<p>a. Cut semithin sections (1-2 µm)</p> <p>b. Affix to a poly-L-Lysin coated slide</p> <p>c. Heat briefly on a slide warmer, 1-2 min</p> <p>d. Incubate in 3% (w/v) BSA, 50 mM Glycin, 1% PBS, 10 min</p> <p>e. Rinse 3x in 1% PBS, 1 min each</p> <p>f. Incubate with mouse anti-callose antibody (1:400 in 1% BSA, 0.02% Tween, 1% PBS), 90 min at 37 °C</p>

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| 4 Immunolocalization | <p>g. Incubate with secondary anti-mouse Alexa Fluor® 594 antibody (1:500 in 1% BSA, 0.02% Tween, 1% PBS), 1h at 37 °C</p> <hr/> <p>h. Rinse 3x in 1% PBS, 1 min each</p> <hr/> <p>i. Incubate in 10 mM calcofluor, 10 min</p> <hr/> <p>j. Rinse with 1% PBS</p> <hr/> <p>k. Air dry and mount with Citifluor Antifade</p> |
| 5 Imaging LSM | <p>a. Cut semithin sections (1-2 µm) or image the intact bloc face</p> <hr/> <p>b. Acquire overview and high magnification images (e.g. 5x and 63x water immersion)</p> |
| 6 Imaging 3D-SIM | <p>a. 3D-SIM is performed on semi and ultrathin sections with a 100x 1.4NA oil immersion objective</p> |
| 7. Imaging TEM | <p>a. Cut ultrathin sections, ~60 nm</p> <hr/> <p>b. Stain in 1% (w/v) aqueous uranyl acetate and Reynolds lead citrate</p> <hr/> <p>c. Image in TEM</p> |
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References:

Bell K., Mitchell S., Paultre D., Posch M., and Oparka K.: Correlative Imaging of Fluorescent Proteins in Resin-Embedded Plant Material, *Plant Physiology*, 2013, (161), pp. 1595–1603

1.3 Protocols for correlative microscopy on vitrified samples (cryo LM/EM)

1.3.1 Sample: Brain hippocampal neuronal cultures

Imaging: Cryo widefield → Cryo TEM

Primary hippocampal neuronal cultures from E18 Sprague-Dawley rats grown on EM gold finder grids were fluorescently labeled for LM and afterwards plunge frozen for cryo EM and cryo tomography.

Cultured primary hippocampal neuronal cultures grown on TEM grids (Imaging: cryoLM → TEM)

1	Labels	Synaptogreen C4
	Fluorescent Labeling	Added to the live culture
2	Cell Culture	<ol style="list-style-type: none"> Sterilize gold finder grids coated with Quantifoil in ETOH for 10 min Coat grids with 1mg/ml poly-L-Lysine for 1 day Wash in D-MEM Place in D-MEM with B27 supplement Plate dissected neurons onto EM grid in culture dishes
3	LM Live Imaging	<ol style="list-style-type: none"> Add 15 μM FM1-43 equivalent (synaptogreen C4) in Hank's Balanced Salt Solution (HBSS, containing 50 mM KCL, 2 mM CaCl_2 and 10 μM CNQX) to the culture for 90 s Incubate in 15 μM FM1-43 equivalent in HBSS at 30-35 $^\circ\text{C}$, for 60 s Induce depolarization unloading of FM1-43 by 60 s incubation in HBSS containing 50 mM KCl, 2 mM CaCl_2, 10 μM CNQX Image grids in HBSS in glass bottom culture dishes at 35 $^\circ\text{C}$, record Z-stacks to compensate for bent grids
4	Cryo Fixation	<ol style="list-style-type: none"> 15 nm BSA tracer gold was spun down at 13,000 rpm for 15-30 min Discard supernatant and resuspend the pellet in HBSS Apply 3 μl to the grids as fiducial markers Blot excess liquid away Plunge freeze in liquid ethane (cooled by nitrogen)
5	Cryo Imaging	<ol style="list-style-type: none"> Acquire additional data on a cryo LM Acquire high resolution data on a cryo EM

Note: Correlation and the transformation of the coordinates between the datasets was implemented in a custom software and performed on a landmark based approach, e.g. finder grid structures. Please refer to the paper for the complete explanation.

References:

Lucić V., Kossel A.H., Yang T., Bonhoeffer T., Baumeister W. and Sartori A.: Multiscale imaging of neurons grown in culture: from light microscopy to cryo-electron tomography. *J Struct Biol*, 2007 (160), 146-156

1.3.2 Sample: HIV-1 (host-cell interaction with HeLa)

Imaging: Cryo LM → Cryo electron tomography

Infection of HeLa cells with HIV-1 is visualized in confocal light microscopy and cryo light microscopy to correlate viral particle location with cryo electron tomography.

HIV host cell interaction with HeLa cells (Imaging: live cell LM → cryo LM → TEM)

1 Labels	GFP
Fluorescent Labeling	HIV-1 containing GFP-Vpr
2 Cell Culture	<ol style="list-style-type: none"> Sterilize gold R2/2 Quantifoil finder grids under UV light for 2 h Coat with 50 µg/ml fibronectin Culture HeLa cells onto gold R2/2 Quantifoil finder grids in glass bottom culture dishes Monitor cells for 20 h by collecting DIC images Infect cells in culture medium with 20 µl of VSV-G pseudotyped HIV-1 Allow viral particles to incubate for 20 min at room temperature to facilitate attachment for the initial correlative analysis. Wash with prewarmed fresh DMEM Incubated for 2 h
3 LM Live Imaging	<ol style="list-style-type: none"> Acquire time-lapse confocal live cell imaging with a 60x, 1.35 NA oil immersion objective Acquire confocal stacks 40 min after addition of GFP-labeled viral particles
4 Cryo Fixation	<ol style="list-style-type: none"> Apply 4 µl of 15 nm gold beads immediately after live imaging Blot grids with filter paper Plunge into liquid ethane for vitrification
5 Cryo Imaging LM	<ol style="list-style-type: none"> Load vitrified grids into cryo LM stage Acquire overview images with the cryo LM Acquire desired image data
6 Cryo Imaging EM	<ol style="list-style-type: none"> Transfer grids to the cryo EM Acquire low magnification overview images for correlation Acquire high resolution image data and tomograms

References:

Jun S., Ke D., Debiec K., Zhao G., Xin Meng, Ambrose Z., Gibson G. A, Watkins S. C. and Zhang P.: Direct visualization of HIV-1 with correlative live-cell microscopy and cryo-electron tomography. *Structure*, 2011 (19), 1573-1581

2 Correlating Light Microscopy and Scanning Electron Microscopy

2.1 Fluorescent imaging followed by resin embedding and post labeling

2.1.1 Sample: HUVEC in cell culture on Aclar

Imaging: WF/LSM → FIB-SEM, TE

HUVECs are grown on Aclar with user-defined markings. For the method on introducing the patterns into the Aclar please refer to the original publication. The authors of this publication also used different approaches for correlative microscopy based on gridded Aclar, cell morphology, or low resolution fluorescence.

HUVEC on gridded Aclar (Imaging: live cell LM → cryo LM → FIB-SEM)

1 Gridded Aclar	<ul style="list-style-type: none"> a. Refer to publication for the detailed procedure how to prepare the gridded Aclar b. In brief, a pattern is printed at high resolution and used as a guide c. A grid is imprinted with the tip of a needle under a stereomicroscope using the printed grid as reference
2 Cell Culture	<ul style="list-style-type: none"> a. Attach Aclar to the glass bottom of cell culture dishes with Matrigel b. Sterilize the culture dish with the Aclar with UV light c. Culture HUVEC on the gridded Aclar in the cell culture vessels

HUVEC on gridded Aclar correlation based on cell morphology

1 Labels	No labels
Fluorescent Labeling	–
2 Live Cell Imaging	<ul style="list-style-type: none"> a. Monitor the HUVEC cell culture on the gridded Aclar b. Record grid locations of interest and cell shape with phase contrast
3 Fixation	<ul style="list-style-type: none"> a. Fixation is performed with aldehydes b. Postfixation with OsO₄ c. Tannic acid mediated osmium impregnation d. Dehydration e. Immerse Aclar pieces in a drop of fresh EPON on a polyethylene plate f. Divide with a GEM blade under a stereomicroscope g. Cut regions of interest according to grid locations
4 Embedding	a. Embed according to Jimenez et al. (2009) J. Struct. Biol. 166, 103–106.
5 Trimming	<ul style="list-style-type: none"> a. Trim the block down to the Aclar piece b. Peel off a corner of the Aclar piece with forceps. This results in a flat blockface with the grid imprinted on the blockface

6 EM or FIB-SEM	a. Prepare the block for TEM (serial sections) or FIB-SEM
7 FIB-SEM	a. Attach the block to the SEM holder with carbon cement b. Sputter coat with 3 nm platinum/palladium c. Grid lines should be plainly visible in a low kV electron beam d. Relocate the regions of interest using the grid e. Increase the beam current to 20 kV and use a BSE detector for imaging, cell outlines should be visible and cells of interest can be traced back based on the morphology f. Use the FIB to mill into the cells of interest

HUVEC on gridded Aclar correlation with low resolution based fluorescent labels

1 Labels	Low Density Lipoprotein with Oregon green, antibody conjugated Alexa Fluor® 555
Fluorescent Labeling	Oregon green was added to the cell culture. Alexa Fluor® 555 antibodies are used during immunolabeling
2 LM Imaging	a. Two approaches for fluorescent labels are performed b. HUVECs are allowed to internalize LDL-Oregon green <ol style="list-style-type: none"> i. Incubate cells in culture with LDL-Oregon green for 45 min ii. Wash cells to remove non-internalized LDL-Oregon green iii. Fix with 4% PFA in 0.2 M HEPES buffer pH 7.3, 30 min at room temperature iv. Rinse cells in PBS v. Perform confocal laser scanning microscopy of the cells on gridded aclar. Used objectives are 10x/0.3 NA and 40x/1.2NA C-Apo water immersion. The 10x objective was used for overview images of the grid locations. vi. In addition phase contrast images are acquired to provide additional morphology information c. HUVECs are immunolabeled: <ol style="list-style-type: none"> i. Fix with 4% PFA in 0.2 M HEPES buffer pH 7.3, 30 min at room temperature ii. Rinse cells with PBS iii. Quench and permeabilize the sample in one step with 0.5% BSA, 0.045% cold water fish gelatin, 50 mM NH₄Cl, 0.1% saponin in PBS, 30 min at room temperature iv. Incubate with 2 µg/ml rabbit anti-caveolin in the cocktail above, 1h at room temperature v. Wash thoroughly with PBS vi. Incubate with a mix of Protein A-gold 5 nm/ anti rabbit antibody-Alexa Fluor® 555 vii. Wash cells in PBS viii. Perform confocal laser scanning microscopy of the cells on gridded aclar. Used objectives are 10x/0.3 NA and 40x/1.2NA C-Apo water immersion. The 10x objective was used for overview images of the grid locations. ix. In addition, phase contrast images are acquired to provide additional morphology information

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| 3 Fixation | <ul style="list-style-type: none">a. Fixation is performed with aldehydesb. Postfixation with OsO₄c. Tannic acid mediated osmium impregnationd. Dehydratione. Immerse Aclar pieces in a drop of fresh EPON on a polyethylene platef. Divide Aclar pieces with a GEM blade under a stereomicroscopeg. Cut regions of interest according to grid locations |
| 4 Embedding | <ul style="list-style-type: none">a. Embed according to Jimenez et al. (2009) J. Struct. Biol. 166, 103–106. |
| 5 Trimming | <ul style="list-style-type: none">a. Trim the block down to the Aclar pieceb. Peel off a corner of the Aclar piece with forceps. This results in a flat blockface with the grid imprinted on the blockface |
| 6 EM or FIB-SEM | <ul style="list-style-type: none">a. Prepare the block for TEM (serial sections) or FIB-SEM |
| 7 FIB-SEM | <ul style="list-style-type: none">a. Attach the block to the SEM holder with carbon cementb. Sputter coat with 3 nm platinum/palladiumc. Grid lines should be plainly visible in a low kV electron beamd. Relocate the regions of interest using the gride. Increase the beam current to 20 kV and use BSE contrast for imaging, cell outlines should be visible and cells of interest can be traced backf. Use the FIB to mill into the cells of interest |
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References:

Jiménez N., Van Donselaar E. G., De Winter D. A. M., Vocking K., Verkleij A. J., Post J. A. and Aclar G.: preparation methods and use for correlative light and electron microscopy of cell monolayers by TEM and FIB-SEM. J Microsc, 2010 (237), 208-220

2.1.2 Sample: Cytoskeleton of mouse melanoma cell B16F1

Imaging: LM → SEM

Protocol for preparing mouse melanoma cell cytoskeleton for correlative light and scanning electron microscopy on coverslips coated with a gold finder pattern. Fluorescent imaging was performed on the live cell during cell culture.

Extraction of cytoskeleton from mouse melanoma cells (imaging: LM → SEM)

1 Labels	GFP, Phalloidin
Fluorescent Labeling	Transfected with EGFP-fascin
2 Preparation of Locator Coverslips	<ul style="list-style-type: none"> a. Put one or two finder grids in the center of 22x22 mm glass coverslips b. Evaporate gold onto the coverslips in a suitable evaporator (coating should be clearly visible as purple transparent deposit) c. Remove grids and bake coverslips overnight at 160 °C
3 Cell Culture Chambers	<ul style="list-style-type: none"> a. Gold coated coverslips can be mounted onto the hole in the bottom of 35 mm tissue culture dishes b. Smooth edges of the hole before mounting c. Apply a thin line of vacuum grease along the edges (use as little grease as possible required to prevent leakage) d. Mount the coverslip with the gold coated side facing upwards into the tissue culture vessel e. Press coverslip firmly onto the edges in circles and get rid of all possible air bubbles f. Sterilize with UV light
4 LM Imaging	<ul style="list-style-type: none"> a. Imaging with the desired mode of LM can be done directly in the cell culture vessels through the coverslip that is glued to the bottom b. Record grid position during image acquisition c. The following preparation protocols can be started directly on the LM stage in the cell culture vessel
5 Preparation of Cytoskeleton	<ul style="list-style-type: none"> a. Prepare the following solutions: <ul style="list-style-type: none"> i. PEM buffer: 100 mM PIPES, pH 6.9, 1 mM MgCl₂, 1 mM EGTA ii. Extraction solution: 1% Triton X-100, 4% PEG in PEM buffer supplemented with 2 μM Taxol and/or 2 μM phalloidin (add taxol or phalloidin in DMSO before use) iii. Na-cacodylate stock: 0.2 M Na-cacodylate, pH 7.3 iv. Glutaraldehyde: 2% glutaraldehyde in 0.1 M Na-cacodylate (store at 4 °C, use within a week) v. Tannic acid: 0.1% aqueous tannic acid (prepare fresh, use within a day) vi. Uranyl acetate: 0.1% aqueous uranyl acetate b. Exchange cell medium with prewarmed 37 °C PBS directly on the LM stage after LM observations are complete c. Discard PBS and immediately add extraction solution and incubate for 3-5 min d. Rinse cells with PEM buffer 2-3x, 1 min each

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- 5 Preparation of Cytoskeleton**
- e. Add glutaraldehyde, incubate for at least 20 min at room temperature, proceed with step “f” or continue with immunolabeling
 - f. Remove glutaraldehyde and add tannic acid, incubate for 20 min at room temperature
 - g. Rinse 3x in distilled water, incubate 5 min in the last rinse
 - h. Remove water, add uranyl acetate, incubate 20 min at room temperature
 - i. Replace uranyl acetate with distilled water
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- 6 Immunolabeling**
- a. Prepare following solutions:
 - i. Na-borohydrate (NaBH_4): 2 mg/ml NaBH_4 in PBS
 - ii. Primary antibody: estimate the required concentration for your sample through LM experiments
 - iii. Buffer A: 20 mM Tris-HCL, pH 8.0, 0.5 M NaCl, 0.05% Tween
 - iv. Buffer A (0.1% BSA), Store at 4 °C for 1 month
 - v. Buffer A (1% BSA), Store at 4 °C for 1 month
 - vi. Secondary antibody: colloidal gold-conjugated secondary antibody, dilutions 1:5 – 1:10 in Buffer A with 1% BSA
 - b. After glutaraldehyde fixation from the “Preparation of cytoskeleton”, wash the cells with PBS, 2x brief rinses, last exchange incubate in PBS for 5 min
 - c. Quench auto-fluorescence in the sample with NaBH_4 , 10 min at room temperature (shake of bubbles occasionally)
 - d. Rinse 3x with PBS, last exchange incubate for 5 min
 - e. Remove PBS with cotton swabs, leave only a small wet area where the locator grid patterns are located on the coverslip
 - f. Apply primary antibody, incubate 30-45 min at room temperature
 - g. Rinse 3x with PBS, last exchange incubate for 5 min
 - h. Rinse 1x with Buffer A (0.1% BSA)
 - i. Remove PBS with cotton swabs as done above
 - j. Apply secondary antibody with colloidal gold-conjugated, incubate overnight at room temperature in a sealed dish in moist conditions.
 - k. Rinse 3x with Buffer A (0.1% BSA), last exchange incubate for 5 min
 - l. Fix with glutaraldehyde, tannic acid and uranyl acetate (see above)
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- 7 Critical Point Drying (CPD)**
- a. Prepare following solutions:
 - i. Graded ethanol: 12%, 20%, 40%, 60%, 80%
 - ii. Uranyl acetate in ethanol: 0.1% uranyl acetate in 100% ethanol
 - iii. Dried ethanol: 100% ethanol dried over a molecular sieve
 - b. If oil objectives were used, for LM, remove the immersion oil from the bottom with ethanol
 - c. Detach coverslip from the cell culture vessel and quickly transfer it into a water filled petri dish. Some vacuum grease will remain on the lower side of coverslip; gently press the coverslip down and make sure the grease doesn't contaminate the central area of coverslip
 - d. Use a diamond pencil to cut of the greased edges, retain a 6-8 mm central piece
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7 Critical Point Drying (CPD)

- e. Place a specimen holder for CPD into a beaker filled with water
- f. Cut lens tissue into pieces that fit the size of the holder
- g. Put a piece of lens tissue on the bottom of the holder
- h. Place the coverslip onto the tissue, in this way load all your coverslips until the holder is full (up to 12 coverslips with 6-8 mm) with lens tissue as spacers. Put the lid on loosely to prevent the last sheet of lens tissue from floating away
- i. Add 10% ETOH into a 50 ml beaker, put the holder quickly from the water onto a wirescaffold above the magnetic stir bar. Stir for 5 min
- j. Prepare another beaker with 20% ETOH the same way, transfer the holder, stir for 5 min
- k. Repeat this for 40%, 60%, 80% and twice for 100% ETOH, two sets of beakers are enough and can be alternated between steps
- l. After the last step in 100% ETOH, repeat twice with dry 100% ETOH, for 5 min
- m. Fill the specimen chamber of the CPD device with enough dried 100% ETOH to cover the holder
- n. Place holder into the chamber, close it and open the CO₂ supply
- o. Cool down to 10-15 °C, and maintain the temperature until the heating step
- p. Slightly open the exhaust valve for 30 s, keep the inlet valve open, repeat 10 times, every 5 min
- q. Turn on the heat (to raise pressure and temperature over the critical point), then slowly release the pressure by opening the exhaust valve. (A fast decrease in pressure and temperature may cause condensation and ruin the dry samples)
- r. Remove holder from the CPD device and place it in a sealed desiccated container

8 SEM Imaging

- a. Samples can be rotary shadowed, metal or carbon coated for SEM
 - b. In addition, Platinum replicas can be produced for TEM investigation, please refer to the original publication of Platinum replica generation
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References:

Tatyana M., Borisy G. G.: Correlative Light and Electron Microscopy of the Cytoskeleton, ELECTRON MICROSCOPY, Elsevier, 2005

2.1.3 Sample: Mouse cortical neurons

Imaging: Two-photon LSM → FIB-SEM

Protocol for in vivo two-photon imaging of cortical neurons in the mouse brain. Additionally, laser marks for correlation with FIB-SEM tomography are introduced using the 2-photon laser. The volumes of interest are then prepared for FIB-SEM tomography and correlated with the help of the laser markings.

Mouse cortical neurons – Imaging: two-photon LSM, (Imaging: two-photon LSM → FIB-SEM)

1 Labels	GFP, tdTomato
Fluorescent Labeling	Transgenic mice expressing GFP in pyramidal cells (line M), or tdTomato in cortical inhibitory interneurons
2 Mouse Preparation	<ol style="list-style-type: none"> In vivo imaging through a cranial window is described in Holtmaat A et al. (2009) Nat Protoc 4: 1128–1144 Produce a small craniotomy spanning approx. 3 mm above the somatosensory cortex Cover with a coverslip (#1) thus generating an optical window Seal the window with dental cement Start imaging after approx. 7 days
3 In Vivo Imaging	<ol style="list-style-type: none"> Anesthetize animals with an intraperitoneal injection of ketamine and xylazine (0.13 mg/g and 0.01 mg/g body weight) Image bright field overview images, low and high resolution images of axons and dendrites for correlation
4 Fixation	<ol style="list-style-type: none"> Fix the anesthetized animals by cardiac perfusion of: <ol style="list-style-type: none"> 10 ml isotonic PBS Follow immediately with 200 ml mix of 2.5% glutaraldehyde, 2% PFA in 100 mM phosphate buffer, pH 7.4, 12 ml/min with a peristaltic pump Remove brain 2 h after perfusion Cut 60 µm thick vibratome sections through the somatosensory cortex
5 LM Imaging	<ol style="list-style-type: none"> Image sections at 20x with widefield and phase contrast to highlight vasculature Superimpose the images with the vascular pattern Use this information to identify the regions that were previously imaged
6 Laser Marking	<ol style="list-style-type: none"> Mount the sections temporarily in PBS in an imaging chamber Use a parafilm space under the coverslip Perform laser marking of the selected dendrites according to Bishop D. et. al. Nat. Methods 8: 568–570 Typically use 512 to 2500 laser line scans at $\lambda = 810$ nm and ~300 mW, 2 ms/line until autofluorescence appears Use larger asymmetric markings to later identify the orientation Collect image stacks before and after laser marking to visualize the exact locations of the markings

7 EM Processing

- a. Remove the section from the imaging chamber
- b. Wash in Na-cacodylate buffer, 0.1 M, pH 7.4
- c. Postfix in 1.5% potassium ferrocyanide , 1% OsO₄ in cacodylate buffer, 40 min
- d. Incubate in 1% OsO₄ in cacodylate buffer, 40 min
- e. Incubate in 1% aqueous uranyl acetate, 40 min
- f. Dehydrate in a grade ETOH series
- g. Infiltrate with Durcupan resin overnight
- h. Flat embed in fresh Durcupan between glass slides
- i. Polymerize at 65 °C, 24 h

8 Block Preparation

- a. Separate resin embedded samples from the glass using a light microscope to identify the laser markings
- b. Mark the identified positions (e.g. with a laser dissecting microscope)
- c. Glue the sample to a blank 1 mm thick slab of resin
- d. Identify the depth of the two-photon laser marks in a light microscope (ideally with a calibrated stage that shows the height)
- e. Trim the block to around 5 µm distance between the block face and the 2 photon laser marks
- f. Use a laser dissecting microscopy to replicate the two-photon laser marks onto the blockface surface
- g. Photograph the final block face to show the position of the two-photon laser marks and the etched surface marks
- h. Mount the block onto an 45° inclined SEM aluminum stub using conductive carbon paint
- i. Coat with a 30 nm thick layer of gold

9 FIB-SEM

- a. A ~1 µm thick layer of platinum was deposited onto the block in the SEM above the region of interest to reduce milling artifacts
- b. Orient the block using low magnification and secondary electrons (5 kV, 0.5 nA) using the surface laser marks
- c. Mill and initial imaging face 100x100 µm with an ion beam of 13-27 nA at 30 kV to remove a narrow band of resin
- d. Image with backscattered electrons to see the tissue inside
- e. Put a protective layer of 1 µm carbon onto the block face above the region of interest
- f. Finally mill the area and collect the image series (see details in the publication)

References:

Maco B., Holtmaat A., Cantoni M., Kreshuk A., Straehle, C. N., Hamprecht F. A., Knott G. W.: Correlative In Vivo 2 Photon and Focused Ion Beam Scanning Electron Microscopy of Cortical Neurons, PLOS ONE, www.plosone.org, 1 February 2013, Volume 8, Issue 2, e57405

2.2 Protocols for preserving fluorescence in resin embedded samples

2.2.1 Sample: *C. elegans*

Imaging: STED, PALM → SEM

Localization of histones, a mitochondrial protein, and a presynaptic dense projection protein in electron micrographs of thin sections from *C. elegans* with correlative superresolution light microscopy.

C. elegans, stably expressing fluorescent tags (Imaging: PALM → SEM)

1 Labels	Citrine, tdEos, Dendra
Fluorescent Labeling	Stable expression of desired fusion proteins
2 Fixation (High-Pressure-Freezing)	<ol style="list-style-type: none"> Prepare fixative and freeze substitution media; 95% anhydrous acetone, 5% water. As fixative 0.1-2% PFA, 0.1%-1% glutaraldehyde, 0.1% acrolein, 0.001%-0.5% OsO₄, 0.1% KMnO₄ is prepared in cryogenic vials, freeze in liquid nitrogen prior to use Place animals into 100 µm deep type A specimen carriers (OP50 or HB101) filled with bacteria Freeze in a high pressure freezer (e.g. BAL-TEC HPM 010)
3 Freeze Substitution	<ol style="list-style-type: none"> Transfer specimens into cryogenic vials containing the freeze substitution media and fixatives Transfer the vials into an automated freeze substitution unit Run the freeze substitution program <ol style="list-style-type: none"> 30 h at -90 °C Heat with 5 °C/h to -20 °C 2 h at -20 °C
4 Infiltration	<ol style="list-style-type: none"> Wash in 95% ethanol for 2 h to remove all fixatives and acetone Different resins (Lowicryl K4M, LR Gold, LR White, glycol methacrylate) are used. Please refer to the publication for detailed information Infiltration with resin was performed at -20 °C: <ol style="list-style-type: none"> 30% resin, 5 h 70% resin, 6 h 95~98%, overnight
5 Polymerisation	<ol style="list-style-type: none"> Please refer to the publication for detailed information on the polymerization of each resin All resin embedding caps are covered with a layer of aclar film to block oxygen Polymerization is carried out for 24 h Polymerized blocks are stored in nitrogen filled vacuum bags at -20 °C, if not sectioned immediately.
6 Ultramicrotomy	<ol style="list-style-type: none"> Cut 70 – 500 nm thick sections Collect sections on precleaned coverslip, #1.5, 18x18 mm for STED, and #1.5 25 mm diameter for PALM

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- 6 Ultramicrotomy**
- c. Coverslip cleaning:
- i. Coverslips for PALM were incubated in Piranha solution (3 parts H_2SO_4 ; 1 part H_2O_2 (30%)). Exercise great care! Mixing is exothermic, temperature of the mixture can reach $>100\text{ }^\circ\text{C}$, violent bubbling can occur that can spray corrosive liquid. Mixture is potentially explosive. Working under a fume hood with personal protective equipment (face shield, acid resistant gloves, lab coat) mandatory!
 - ii. Wash 6x with milliQ water
 - iii. Dip in 100% ETOH to make surface hydrophobic
 - iv. Air dry coverslips
-
- 7 Superresolution Imaging**
- a. STED imaging:
- i. Dilute silica nanoparticles 1:10000 in milliQ water
 - ii. For LR white, $\sim 10\text{ }\mu\text{l}$ of the nanoparticle solution was applied to each coverslip. For glycol methacrylate $10\text{ }\mu\text{l}$ from a 1:500 dilution was used
 - iii. Incubate for 5 min and wash the coverslips afterwards
 - iv. Keep samples under nitrogen until they are dry
 - v. Coverslips are placed in a concave microscope slide filled with water
 - vi. Perform STED imaging
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- b. PALM imaging:
- i. Gold nanoparticles (100 or 250 nm) are diluted 1:10 in filtered milliQ water
 - ii. Apply to coverslips and incubate for 4 min
 - iii. Wash coverslips with filtered milliQ water
 - iv. Mount into coverslip holder
 - v. Perform PALM imaging
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- 8 SEM Imaging**
- a. Stain sections on coverslips with 2.5% uranyl acetate in water, 4 min
 - b. Carbon coat the sections on the coverslips
 - c. Image backscattered electrons (BSE)
-

Note: This study used four different resins: Lowicryl K4M, LR Gold, LR White and glycol methacrylate (GMA). LR White had a pH too acid for most fluorescent proteins. Neutralizing this pH with ethanolamine lead to incomplete polymerization of LR White. Full polymerization could be achieved with 0.025% ethanolamine, which increased the pH to 6.5

Fluorescence is quenched if the embedded samples are left at room temperature for a few days. Storage of embedded samples and sections in nitrogen filled vacuumed bags at $-20\text{ }^\circ\text{C}$ is recommended. Sections of 100 nm preserve fluorescence better than $<100\text{ nm}$ during shipping.

References:

- Watanabe S., Richards J., Hollopeter G., Hobson R. J., Davis W. M., Jorgensen E. M.: (2012) Nano-fEM: Protein Localization Using Photo-activated Localization Microscopy and Electron Microscopy *J Vis Exp.* 3;(70). pii: 3995. <http://www.jove.com/video/3995/nano-fem-protein-localization-using-photo-activated-localization>
- Watanabe S., Punge A., Hollopeter G., Willig K. I., Hobson R. J., Davis M. W., Hell S. W., Jorgensen E. M. Protein localization in electron micrographs using fluorescence nanoscopy. *Nature Methods* 2011; 8:80-84.

2.2.2 Sample: Mitochondrial labeling in mouse fibroblasts

Imaging: iPALM → FIB-SEM

3D superresolution light microscopy (iPALM) of mitochondrial nucleoids

C. *elegans*, stably expressing fluorescent tags (Imaging: iPALM → SEM)

1	Labels	mEos2
	Fluorescent Labeling	Stable expression of desired fusion proteins
2	Fixation	<ol style="list-style-type: none"> Prepare cells for Tokuyasu cryosections according to: Brown TA, et al. (2011), Mol Cell Biol 31: 4994–5010. The general protocol described herein was changed the following way: <ol style="list-style-type: none"> As fixation agent use 4% (w/v) PFA, 2% (w/v) glutaraldehyde in 100 mM sodium phosphate, pH 7.4, 5% (w/v) sucrose
3	Cryosections	<ol style="list-style-type: none"> Cut cryosections with a cryo diamond knife at -80 °C Retrieve cryosections with drops of 1:1 methylcellulose (2% (w/v): sucrose (2.3 M) Place on 25 mm #1.5 coverslips coated with 25x35 nm bare Au nanorods (coating described in Shtengel G., et al. (2009) PNAS, 106:3125–3130)
4	iPALM Imaging	<ol style="list-style-type: none"> Wash sections on coverslips 3x in PBS, 2 min each Treat sections with 0.5% sodium borohydride in 100 mM sodium phosphate buffer, pH 7.4 Wash 2x in PBS, 2 min each Place an 18 mm #1.5 coverslip on top of the other and seal with 5 min epoxy and Vaseline Perform iPALM imaging
5	Poststaining	<ol style="list-style-type: none"> Remove top coverslip Wash 3x in PBS, 2 min each Wash 3x in deionized water, 3 min each Place sections on coverslip upside down on a drop of 0.5% (w/v) uranyl acetate in 1.8% (w/v) methylcellulose on ice, 20 min Wick excess methylcellulose away with a filter paper Air dry the sections on the coverslip Add a drop of cyanoacrylate over the dried sections and centrifuge in a spin coater at 1000 rpm for 20 s Evaporate a 100 nm thick carbon coat onto the sections
6	FIB-SEM Imaging	<ol style="list-style-type: none"> Mill 150 µm wide and 50 µm deep trenches using the FIB the entire volume of interest is imaged Image the upper wall of the trench with SEM (~80x5 µm area) Remove sample/trench in 5 nm steps and repeat imaging until the entire volume of interest is imaged

References:

Kopek B. G., Shtengel G. S., Xu C. S., Clayton D. A. and Hess H. F.: Correlative 3D superresolution fluorescence and electron microscopy reveal the relationship of mitochondrial nucleoids to membranes., Proc Natl Acad Sci, 2012 (109), 6136-6141

2.3 Protocols for correlative microscopy on vitrified samples (cryo LM/SEM/FIB)

2.3.1 Sample: Eukaryotic and procaryotic cells

Imaging: Cryo widefield → SEM/FIB/TEM

Correlative protocols for using vitrified biological samples in a cryo light microscope and prepare the sample through FIB-SEM ion beam milling for cryo TEM. Rat hippocampal neurons grown on EM "Finder" grids and labeled with FM1-43 vital dye are used.

Rat hippocampal neurons (Imaging: WF → TEM)

1 Labels	FM1-43 (in principle all fluorescent dyes are feasible; cryo conditions don't destroy fluorescence)
Fluorescent Labeling	Stable expression of desired fusion proteins, or live staining before vitrification
2 Fixation	<ol style="list-style-type: none"> Plunge freezing of biological material to obtain specimen embedded in vitrified ice on EM grids (high pressure freezing also possible) Transfer the specimen into a cryo light microscopy stage keeping the samples below the devitrification point at all times (-135 °C)
3 Cryo LM Imaging	<ol style="list-style-type: none"> Acquire images and grid positions in cryo widefield light microscope
4 FIB-SEM Processing	<ol style="list-style-type: none"> Mounting the EM grid with the vitrified samples into a cryo-shuttle for FIB-SEM Preparing thin regions of target regions by ion beam milling. Temperature should be kept at -170 °C Transfer to the cryo TEM
5 Cryo TEM	<ol style="list-style-type: none"> Cryo imaging and tomography of the prepared thin region

References:

Rigort A., Villa E., Bäuerlein F. J., Engel B. D., Plitzko J. M.: Integrative approaches for cellular cryo-electron tomography: correlative imaging and focused ion beam micromachining. *Methods Cell Biol.* 2012;111:259-81.

3 Additional References

3.1 Mapping Procedures

3.1.1 Sample: Small invertebrate organism (here *C. elegans*, *Drosophila*)

Imaging: WF, LSM → TEM

Introduction of a mapping system to simplify and speed up retrospective location of the ROI to within 1 μm for larger samples.

General procedure

1 Labels	GFP, RFP
Fluorescent Labeling	Stable expression of desired fusion proteins
2 Preparation	<ol style="list-style-type: none"> a. Embedding of the animals into 4% (w/v) low melting agarose with aclar spacers on a glass slide b. After live imaging punch biopsy is used to transfer the animal from the agarose into high pressure freezing planchettes c. Freeze substitution and EPON (Lowicryl HM20 for immunogold) embedding for 60 h d. Flat embedding into EPON using the same layout as for the agarose embedding e. Carving of surface guides into the resin using a laser microdissector. Lines are carved perpendicular to the long axis of the specimen f. ImageJ is used to make a scaled map of the specimen with the tip of the specimen being used as origin g. Excess resin is trimmed away down to the laser markings with a trimming knife in the ultramicrotome h. The respective ROI is relocated by following the distance information generated in ImageJ and using the microtome feed parameters and section thickness settings of the ultramicrotome
3 Imaging	<ol style="list-style-type: none"> a. Widefield and confocal light microscopy of agarose embedded specimens b. TEM of serial thin sections after freeze substitution, flat embedding and serial sectioning

References:

Kolotuev I., Schwab, Y. and Labouesse M.: A precise and rapid mapping protocol for correlative light and electron microscopy of small invertebrate organisms. *Biol Cell*, 2010 (102), 121-132

3.2 Reviews/Overviews

3.2.1 Overview: Current and New Developments; Tokuyasu Cryosections

Imaging: LSM → TEM [1]

3.2.2 Book: Methods in Cell Biology: Overview of the latest methods in correlative microscopy

Imaging: LM → TEM/SEM/FIB [2]

3.2.3 Protocols: Practical Methods in High-Pressure Freezing, Freeze-Substitution, Embedding and Immunocytochemistry for Electron Microscopy [3]

References:

- [1] Cortese K., Diaspro A. and Tacchetti C.: Advanced correlative light/electron microscopy: current methods and new developments using Tokuyasu cryosections. *J Histochem Cytochem* 2009 (57), 1103-1112
- [2] *Methods in Cell Biology*, VOLUME 111 (2012), *Correlative Light and Electron Microscopy*, Editors: Thomas Müller-Reichert and Paul Verkade, Academic Press, Elsevier
- [3] Morphew M. K.: The Boulder Laboratory For 3-D Electron Microscopy of Cells Dept. of MCD Biology, University of Colorado Boulder, Colorado <http://bio3d.colorado.edu/docs/mmanual.pdf>



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