Journal of Visualized Experiments

Mitochondria and Endoplasmic Reticulum Imaging by Correlative Light and Volume Electron Microscopy

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59750R2
Full Title:	Mitochondria and Endoplasmic Reticulum Imaging by Correlative Light and Volume Electron Microscopy
Keywords:	Mitochondria, ER, APEX, HRP, Correlative light and electron microscopy, 3DEM
Corresponding Author:	Jiyoung Mun Korea Brain Research Institute Daegu, KOREA, REPUBLIC OF
Corresponding Author's Institution:	Korea Brain Research Institute
Corresponding Author E-Mail:	mjy1026@gmail.com
Order of Authors:	Jiyoung Mun
	Minkyo Jung
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Daegu

TITLE:

Mitochondria and Endoplasmic Reticulum Imaging by Correlative Light and Volume Electron Microscopy

AUTHORS AND AFFILIATIONS:

Minkyo Jung[1], Ji Young Mun[1]

[1]Department of Structure and Function of Neural Network, Korea Brain Research Institute, Daegu, South Korea

Corresponding author:
Ji Young Mun (mjy1026@gmail.com)

Email address of co-author:
Minkyo Jung (jungminkyo1@gmail.com)

KEYWORDS:

mitochondria, ER, APEX2, HRP, correlative light and electron microscopy, 3DEM

SUMMARY:

We present a protocol to study the distribution of mitochondria and endoplasmic reticulum in whole cells after genetic modification using correlative light and volume electron microscopy including ascorbate peroxidase 2 and horseradish peroxidase staining, serial sectioning of cells with and without the target gene in the same section, and serial imaging via electron microscopy.

ABSTRACT:

Cellular organelles, such as mitochondria and endoplasmic reticulum (ER), create a network to perform a variety of functions. These highly curved structures are folded into various shapes to form a dynamic network depending on the cellular conditions. Visualization of this network between mitochondria and ER has been attempted using super-resolution fluorescence imaging and light microscopy; however, the limited resolution is insufficient to observe the membranes between the mitochondria and ER in detail. Transmission electron microscopy provides good membrane contrast and nanometer-scale resolution for the observation of cellular organelles; however, it is exceptionally time-consuming when assessing the three-dimensional (3D) structure of highly curved organelles. Therefore, we observed the morphology of mitochondria and ER via correlative light-electron microscopy (CLEM) and volume electron microscopy techniques using enhanced ascorbate peroxidase 2 and horseradish peroxidase staining. An en bloc staining method, ultrathin serial sectioning (array tomography), and volume electron microscopy were applied to observe the 3D structure. In this protocol, we suggest a combination of CLEM and 3D electron microscopy to perform detailed structural studies of mitochondria and ER.

INTRODUCTION:

Mitochondria and endoplasmic reticulum (ER) are membrane-bound cellular organelles. Their connection is necessary for their function, and proteins related to their network have been

described[1]. The distance between the mitochondria and ER has been reported as approximately 100 nm using light microscopy[2]; however, recent super-resolution microscopy[3] and electron microscopy (EM)[4] studies have revealed it to be considerably smaller, at approximately 10–25 nm. The resolution achieved in super-resolution microscopy is lower than EM, and specific labeling is necessary. EM is a suitable technique to attain a sufficiently high-resolution contrast for structural studies of the connections between mitochondria and ER. However, a disadvantage is the limited z-axis information because the thin sections must be approximately 60 nm or thinner for conventional transmission electron microscopy (TEM). For sufficient EM z-axis imaging, three-dimensional electron microscopy (3DEM) can be used[5]. However, this involves the preparation of hundreds of thin serial sections of whole cells, which is very tricky work that only a few skilled technologists have mastered. These thin sections are collected on fragile formvar film-coated one-hole TEM grids. If the film breaks on one gird, serial imaging and volume reconstruction is not possible. Serial block-face scanning electron microscopy (SBEM) is a popular technique for 3DEM that uses destructive en bloc sectioning inside the scanning electron microscope (SEM) vacuum chamber with either a diamond knife (Dik-SBEM) or a focused ion beam (FIB-SEM)[6]. However, because those techniques are not available at all facilities, we suggest array tomography[7] using serial sectioning and SEM. In array tomography, serial sections cut using an ultramicrotome are transferred to a glass coverslip instead of a TEM grid and visualized via light microscopy and SEM[8]. To enhance the signal for backscatter electron (BSE) imaging, we utilized an en bloc EM staining protocol employing osmium tetroxide (OsO_4)-fixed cells with osmiophilic thiocarbohydrazide (TCH)[9], enabling us to obtain images without post-embedding double staining.

Additionally, the mitochondrial marker SCO1 (cytochrome c oxidase assembly protein 1)–ascorbate peroxidase 2 (APEX2)[10] molecular tag was used to visualize mitochondria at the EM level. APEX2 is approximately 28 kDa and is derived from soybean ascorbate peroxidase[11]. It was developed to show the detailed location of specific proteins at the EM level in the same way that green fluorescent protein-tagged protein is used in light microscopy. APEX2 converts 3,3' -diaminobenzidine (DAB) into an insoluble osmiophilic polymer at the site of the tag in the presence of the cofactor hydrogen peroxide (H_2O_2). APEX2 can be used as an alternative to traditional antibody labeling in EM, with a protein localization throughout the depth of the entire cell. In other words, the APEX2-tagged protein can be visualized by specific osmication[11] without immunogold labeling and permeabilization after ultra-cryosectioning. Horseradish peroxidase (HRP) is also a sensitive tag that catalyzes the H_2O_2-dependent polymerization of DAB into a localized precipitate, providing EM contrast after treatment with OsO_4. The ER target peptide sequence HRP–KDEL (lys-asp-glu-leu)[12] was applied to visualize ER within a whole cell. To evaluate our protocol of utilizing genetic tags and en bloc staining with reduced osmium and TCH (rOTO method), using the osmication effect at the same time, we compared the membrane contrast with and without the use of each genetic tag in rOTO en bloc staining**.** Although 3DEM with array tomography and DAB staining with APEX and HRP have, respectively, been utilized for other purposes, our protocol is unique because we have combined array tomography for 3DEM and DAB staining for mitochondria and ER labeling. Specifically, we showed five cells with and without APEX-tagged genes in the same section, which aided in investigating the effect of the genetic modification on cells.

PROTOCOL:

1. Cell culture with patterned grid culture dish and cell transfection with SCO1–APEX2 and HRP–KDEL plasmid vector

1.1. Seed 1 x 10^5 HEK293T cells by placing them into 35-mm glass grid-bottomed culture dishes in a humidified atmosphere containing 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin.

1.2. The day after seeding the cells, when they have grown to 50%−60% confluency, introduce the SCO1–APEX2[10] and HRP–KDEL[12] plasmid to the cells using transfection reagent according to the manufacturer's instructions (SCO1–APEX2 cDNA 0.5 µg + HRP–KDEL plasmid DNA 0.5 µg per 3 µL transfection reagent).

2. Light microscopy of cells growing on patterned culture dishes and DAB staining for APEX2 and HRP

2.1. At 16−24 h after transfection, remove all the culture media and immediately add 250 µL of warm (30−37 °C) fixation solution (**Table 1**) by gentle pipetting. Immediately remove the fixation solution and replace it with 1.5 mL of fresh fixation solution. Incubate on ice for 60 min, and then wash three times for 10 min each in 1 mL of ice-cold 0.1 M sodium cacodylate buffer (**Table 1**).

CAUTION: Aldehyde fumes are extremely toxic. Perform all work under a ventilated fume hood.

2.2. Add 1 mL of cold (0−4 °C) 20 mM glycine solution and incubate for 10 min on ice followed by three washes of 5 min each in 1 mL of cold 0.1 M sodium cacodylate buffer.

2.3. Prepare a fresh 1x DAB solution (3.33 mL of 0.3 M cacodylate solution + 10 µL of 30% H_2O_2 + 5.67 mL of cold water + 1 mL 10x DAB solution).

2.4. Add 500 µL of the freshly prepared 1x DAB solution (step 2.3) and incubate on ice for approximately 5−45 min until a light brown stain is visible under an inverted light microscope (**Figure 1A**).

2.5. Gently remove the DAB solution and rinse three times with 1 mL of cold 0.1 M sodium cacodylate buffer for 10 min each.

2.6. Use a phase-contrast inverted microscope (or a bright-field light microscope) to visualize the DAB staining at a magnification of 100x or higher. Use a marker pen to mark the bottom of the glass where the region of interest (ROI) is located (**Figure 1B,C**).

3. Sample preparation for the EM block

3.1. Perform cell culture and DAB staining as described in steps 2.1–2.6.

3.2. Post-fix the samples with 1 mL of 2% reduced OsO_4 for 1 h at 4 °C.

CAUTION: OsO_4 fumes are highly toxic. Perform all work under a ventilated fume hood.

3.3. Prepare a new TCH solution (**Table 1**) during step 3.2 and pass through a 0.22-µm filter.

CAUTION: TCH fumes are highly toxic. Perform all work under a ventilated fume hood.

3.4. Remove the fixative and rinse three times with 1 mL of distilled water for 5 min each at room temperature (RT).

3.5. Place the cells in 1 mL of previously prepared and filtered TCH solution for 20 min at RT.

3.6. Rinse the cells three times with 1 mL of distilled water for 5 min each at RT.

3.7. Expose the cells a second time to 1 mL of 2% osmium tetroxide in distilled water for 30 min at RT.

3.8. Remove the fixative and rinse three times with 1 mL of distilled water for 5 min each at RT. Add 1 mL of 1% uranyl acetate (aqueous), and leave overnight at 4 °C in the dark.

3.9. Wash the cells three times in 1 mL of distilled water for 5 min each at RT.

3.10. Pre-warm Walton's lead aspartate solution (**Table 1**) in an oven at 60 °C for 30 min.

3.11. Stain the cells with Walton's lead aspartate solution by adding 1 mL of the pre-warmed lead aspartate solution, and then place in an oven for 30 min at 60 °C.

3.12. Rinse the cells three times with 1 mL of distilled water for 5 min each at RT.

3.13. Incubate in a graded series of 2-mL ethanol aliquots (50%, 60%, 70%, 80%, 90%, 95%, 100%, 100%) for 20 min each at RT.

3.14. Decant the ethanol and incubate for 30 min in 1 mL of 3:1 ethanol:low-viscosity embedding mixture medium at RT.

3.15. Remove the medium and add 1 mL of 1:1 ethanol:low-viscosity embedding mixture medium. Incubate for 30 min at RT.

3.16. Remove the medium and add 1 mL of 1:3 ethanol:low-viscosity embedding mixture medium. Incubate for 30 min at RT.

3.17. Remove the medium and add 1 mL of 100% low-viscosity embedding medium and incubate overnight.

3.18. Embed the sample in 100% low-viscosity embedding mixture and incubate for 24 h at 60 °C.

3.19. Prepare 90-nm thick sections using an ultramicrotome.

3.20. Observe the grid under TEM at 200 kV.

4. Serial sectioning and mounting on indium-tin-oxide coated coverslips for SEM imaging

4.1. Substrate preparation

4.1.1. Clean indium-tin-oxide (ITO)-coated glass coverslips (22 mm x 22 mm) by gentle agitation in isopropanol for 30–60 s.

4.1.2. Remove the coverslips, drain off the excess isopropanol, and leave in a dust-free environment until dry.

4.1.3. Treat the ITO-coated glass coverslips by glow discharge using a plasma coater for 1 min.

NOTE: Plasma activating confers a hydrophilic property on the substrate surface. It creates a very thin film of water on the substrate to prevent wrinkle formation in the sections when the section is attached to the substrate.

4.1.4. Insert the ITO-coated glass coverslips into the substrate holder, and place into the knife boat.

4.2. Trimming of the sample block and serial sectioning

4.2.1. Insert the sample block into the sample holder of the ultramicrotome and set into the trimming block.

4.2.2. Use a razor blade to trim away all excess resin around the target position (identified in step 2.6, **Figure 1D–G**). The shape of the block face should be trapezoid or rectangular. The leading edge and trailing edge must be absolutely parallel (**Figure 1H,I**).

4.2.3. Insert the sample holder on the arm of the ultramicrotome and place the diamond knife in the knife holder. Insert the ITO glass coverslips into the ribbon carrier and clamp the carrier with the handle (**Figure 1J**). Set the ribbon carrier into the knife boat and fill the knife boat with filtered distilled water (**Figure 1K**).

4.2.4. Adjust the carrier position with the slide of the knife by carefully pushing the handle of the

holder to set the edge of the ITO glass close to the knife (**Figure 1L**).

4.2.5. After cutting the section, stop the sectioning process, and slowly open the clamping screw of the tube and drain the water boat (flow rate of one drop of water per second).

4.2.6. After completing the ribbon-collection process, remove the substrate with the handle of the clamping device and dry the ribbon (**Figure 1M**).

5. Imaging in the SEM and alignment of the SEM image stack

5.1.1. Mount the ITO-coated coverslip on aluminum stubs with sticky carbon tape. Seal the glass surface and the surface in the stub with sticky carbon tape, and then coat with a 10-nm thick carbon layer (**Figure 1N**).

5.1.2. Observe the ITO-coated coverslip in a field emission SEM at a low acceleration voltage of 5 kV and a suitable working distance for the efficient collection of BSEs.

5.1.3. Import the serial images into the Image J software (Fiji)[13] using the virtual stack option. Open a new TrakEM[14], and import the image stack into TrakEM. Click the right-mouse button and choose the **align** menu.

5.1.4. Then select the image range (from the first image to the last image). Finish the auto-alignment, save the aligned dataset, and choose **export** to compile a flat image from the selected image range (from the first image to the last image). Finally, save the flat image data in AVI format in the Image J main menu.

NOTE: **Supplemental Movie 1** and **Supplemental Movie 2** show the SEM image stack and cropped image stack, respectively.

6. Segmentation of mitochondria and ER from serial images

6.1.1. Start the 3dmod in IMOD[15] software and open image files.

6.1.2. In the ZaP window, draw the contour of the mitochondria and ER using middle-mouse button.

6.1.3. To visualize the segmented volume, open the **Model View** window (**Supplemental Movie 3)**.

REPRESENTATIVE RESULTS:

Figure 1 describes the schedule and workflow for this protocol. The protocol requires 7 days; however, depending on the time spent on SEM imaging, this may increase. For cell transfection, the confluency of the cells should be controlled so as not to cover the bottom of the entire grid plate (**Figure 1A**). A high cell density could prevent the identification of the cell of interest during

light microscope and EM observation. We used genetically tagged plasmids that expressed APEX2 and HRP to select efficiently transfected cells among the numerous cultured cells in the culture dish. We cultured HEK293T cells and confirmed the expression of SCO1-linked APEX2 (mitochondrial intermembrane space [IMS]) and HRP-conjugated KDEL (ER) in co-transfected cells. Under light microscopy, APEX2-transfected cells were stained a brown color, whereas cells without transfection remained unstained (**Figure 1A** and **Figure 2**). This allowed the identification of the transfected target cells, which were then used for correlative light-electron microscopy (CLEM), using DAB staining in a cultured cell population (**Figure 3D–F** and **Figure 4B**). It was helpful to mark the glass bottom (**Figure 1B,C**) to make it easy to identify the location of the target cell during the flat embedding step (**Figure 1E,F**). When the HEK293T cells were treated with an enhanced "double osmium" staining protocol (rOTO), whole cells were stained a dark color (**Figure 1D**). After removing the gridded coverslip from the culture dish, we identified the target location on the block surface under a stereo microscope (**Figure 1G**). We trimmed the cells in the ROI in a trapezoid shape, and the leading and trailing edges were made parallel (**Figure 1H,I**). To implement mitochondria and ER network reconstruction, we used a large diamond knife with a large water boat to serially section SCO1–APEX2 and HRP–KDEL-expressing HEK293T cells (**Figure 1J–L**). Serial 90-nm thick ribbon sections were successfully attached to the ITO-coated glass coverslip (**Figure 1M**), and the surface was coated with 10-nm thick carbon for observation via SEM (**Figure 1N**).

SCO1–APEX2 and HRP–KDEL proteins generate highly dense electronic signals derived from DAB conversion that are detectable in TEM (**Figure 3**) and SEM (**Figure 4**). The dark stain generated by SCO1–APEX2 was observed exclusively in the IMS and not in the matrix space of mitochondria (**Figure 3D**). Co-transfected cells (the left cell in **Figure 3D,E**) with both SCO1–APEX2 and HRP–KDEL plasmids expressed a highly dense electron signal in mitochondrial IMS and ER; however, we observed no ER staining in cells that were transfected only with SCO1–APEX2 (the right cell in **Figure 3D,F**). For serial images using SEM, first, we created an overview of the whole array image using the BSE detector over a large area (**Figure 4A**). Second, the ROI was placed in the first section and propagated to all other sections (**Figure 4B**). Finally, we visualized the ROI containing five target cells with 5-nm image pixels (**Figure 4C**). Zoomed-in images revealed detailed subcellular structures (**Figure 4D**) such as Golgi apparatus, mitochondria, nuclei, and ER. The serial images clearly showed that ER–mitochondria contacts were occurring on different z-planes (**Supplemental Movie 2** and **Supplemental Movie 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Sample preparation workflow for SEM and TEM. (**A**) A culture dish containing gridded coverslips was seeded with cells and stained with DAB. (**B**,**C**) After DAB staining, a marker pen was used to mark the bottom of the glass where the target cells were located. (**D**) After O_sO_4 staining, the cells become a dark color. (**E,F**) Polymerase chain reaction (PCR) tubes (or any type of embedding capsules) were used to easily make an EM block that contained the marked positions. (**E**) Top view. (**F**) Bottom view. (**G**) A low-magnification stereo microscopy image of the surface of an EM block. (**H**) The ROI is in the middle of the flat surface. (**I**) A higher-magnification stereo microscopy image of the rectangular ROI. (**J**) ITO-coated glass coverslips (white asterisk)

are inserted into the ribbon carrier (blue arrow), and the carrier is clamped by turning the handle clockwise. (**K,L**) The handle of the holder is carefully pushed, and the carrier is adjusted to position the edge of the ITO-coated glass coverslip close to the knife by sliding. (**M**) The ribbons are attached to the ITO-coated glass coverslips. (**N**) The ITO-coated glass coverslips are attached to the SEM stubs, and the residual glass is sealed with sticky carbon tape and coated with a 10-nm carbon thread layer. White asterisks indicate the ITO-coated glass coverslip, and black asterisks indicate the carbon tape.

Figure 2: Inverted phase-contrast microscopy image of cultured HEK293T cells stained with DAB. (**A**) Staining of cells with DAB only (without O_sO_4 staining). White arrows indicate the unstained cells, and black arrows indicate DAB-stained cells. (**B**) Higher-magnification image of the ROI.

Figure 3: TEM imaging of HEK293T cells exhibiting the targeted mitochondrial IMS (SCO1–APEX2) and ER (HRP–KDEL). (**A–C**) Untransfected HEK293T cells showing the double-membrane of mitochondria (M) and endoplasmic reticulum (ER). (**D–F**) APEX2 and HRP catalyze the polymerization of DAB into a local precipitate, which is subsequently stained with electron-dense OsO_4. A dark contrast is apparent in the mitochondrial IMS (black arrowhead) and ER (black arrow); however, cells that were not transfected with HRP–KDEL exhibit unstained ER (white arrow). Scale bars: 1 µm.

Figure 4: Serial SEM imaging of HEK293T cells exhibiting the targeted mitochondrial IMS (SCO1–APEX2) and ER (HRP–KDEL). (**A**) Overview of the serial section ribbons observed using the BSE detector. (**B**) Correlation of low-magnification image (inset) with high-magnification BSE image (white dotted-line box indicates the ROI of DAB-stained cells). (**C**) High magnification of the ROI target cells with 5-nm image pixels. (**D,E**) A dark contrast is apparent in the mitochondrial IMS and ER but not the Golgi apparatus. (**F**–**I**) ER–mitochondria contacts (white arrow) occur on different z-planes. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; G, Golgi apparatus.

Table 1: Solution recipes.

Supplemental Movie 1: SEM image stack. Fiji[13] with TrakEM[14] software was used to align 91 images. The original aligned data set is 11 GB. To downsize the stack, resized and cropped image set was used.

Supplemental Movie 2: Cropped image stack. To visualize mitochondria, ER, and their contact sites in detail, images were cropped from original data set (5 nm/pixel). Scale bars: 1 µm.

Supplemental Movie 3: 3D Reconstruction of mitochondria and ER. For 3D visualization, the contour of mitochondria and ER was segmented and visualized using IMOD[15] software. Mitochondria were visualized as long tubular structures (red), and the ER networks (green) showed their complicated morphology. Yellow represented a large surface area of contact site between mitochondria and ER in different z-planes.

DISCUSSION:

Determining the cellular localization of specific proteins at a nanometer resolution using EM is crucial to understand the cellular functions of proteins. Generally, there are two techniques to study the localization of a target protein via EM. One is the immunogold technique, which has been used in EM since 1960, and the other is a technique using recently developed genetically encoded tags[16]. Traditional immunogold techniques have employed antibody-conjugated gold particles or quantum dots to show the location of the labeled protein. However, due to the requirement for high-quality antibodies and the penetration efficiency of antibodies affected by resin and fixative, this technique is significantly limited[17]. Specifically, because immunogold labeling is predominantly restricted to the surface of an ultrathin section without en bloc metal staining and strong osmium fixation, this technique is not directly applicable to modern 3DEM[18]. To use recent 3DEM methods, including SBEM and array tomography, with protein labeling, we utilized genetically encoded EM tags in this protocol. Genetically encoded tags do not require permeabilization, technically demanding ultra-cryosectioning, and immunostaining of individual sections because they localize to the site of interest prior to fixation.

The procedures for sample preparation in 3DEM generally include a combination of common chemical fixation and heavy metal staining methods because cells are composed mainly of C, H, O, and N, requiring staining with heavy metals to acquire contrast under EM[9]. Therefore, we employed reduced osmium fixation and metal staining to enhance contrast and conductivity for serial imaging. The procedure to stain samples before sectioning, known as en bloc staining, has been reported as an essential step for 3DEM methods such as SBEM and FIB-SEM[19]. We confirmed that the en bloc-stained cells in our protocol demonstrated clear SCO1–APEX2 and HRP–KDEL EM contrast exclusively in the mitochondrial IMS and ER, respectively, in TEM (**Figure 3**). Furthermore, the SEM images from 90-nm serial sections revealed a clear contrast in both organelles (**Figure 4**). Notably, the enhanced contrast by en bloc staining was distinctly distinguishable from the DAB signal, and the contrast and conductivity resulted in good-quality serial images (**Figure 4**). Additionally, this high contrast aids the facilitation of subsequent tasks such as alignment and segmentation with three-dimensional (3D) image software.

In recent years, volume electron microscopy techniques (dik-SBEM, FIB-SEM, and array tomography) have answered biological questions that required the observation of a large field of view and a 3D view. Dik-SBEM and FIB-SEM do not involve the physical handling of sections, so time consuming for alignment of images can be reduced. However, the sample has to be destroyed to obtain serial images, and the field of view is smaller than that of array tomography. Serial SEM imaging using array tomography is employed increasingly as an alternative to TEM serial sectioning, and the major advantage of this technique is its non-destructive manner and large field of view. Unlike other 3DEM techniques such as dik-SBEM and FIB-SEM, sections can be stored on a coverslip, an ITO-coated coverslip, a silicon wafer, or a tape and can be repeatedly imaged[7].

APEX2 is easy to use and can give a wide range of staining densities without special equipment, unlike mini singlet oxygen generator[20] or fluorescent protein[21] techniques, generating DAB precipitation via a photooxidation. Its variable application has been tested in several cellular

organelles including nucleus, plasma membrane, mitochondria matrix, mitochondrial cristae, ER, tubulin, and actin in COS 7 and HEK293T cell[22]. However, there are some limitations and several checkpoints for the use of genetically encoded tagging in electron micrographs. The expression levels of the exogenous genes should be controlled to achieve reasonable staining at EM level because if the expression of the genetic tag is too high, it may induce false-positive signals and perturb the ultrastructure of cells via membrane rupture and subcellular organelle aggregation[23]. Another possible problem is DAB overstaining, which has been reported to cause blurriness and membrane destruction[22]. To ensure the appropriate expression of genes, DAB-stained cells were compared with unstained cells using both light microscopy and EM (**Figure 2** and **Figure 3**). Additionally, the fixation level should be well regulated to ensure that the endogenous oxidases are fully inactive. To prevent any artifacts from endogenous oxidases during processing, we fixed a monolayer of cells instead of using detached and pelleted cells[24]. This also helped to identify the cells of interest when we checked the DAB signal under light microscopy. Thus, our results indicate that the staining and fixation of cell monolayers are useful for CLEM using DAB staining (**Figure 2**). The serial images with array tomography and 3D model revealed that the ER–mitochondria contact sites occur on different z-planes (**Supplemental Movie 2** and **Supplemental Movie 3**). The image produces a complete 3D visualization of the mitochondrial and ER networks in whole cells (**Supplemental Movie 1**). It suggests 3D volume analysis is essential for quantitative comparison of ER-mitochondria contacts. When studying the complex networks of intracellular organelles, this is an exceptionally useful technique.

In conclusion, this protocol was an efficient combination of CLEM and 3DEM techniques that allowed whole-cell investigation at EM level. Notably, two different tags at the same time and DAB signals in two different organelles were visible in a whole cell. In addition, labeled cells and unlabeled cells in same section can be compared, because of large scale EM. In this protocol, en bloc staining and DAB signals from genetically encoded tags were useful to investigate the interaction between membranous organelles in whole cells. This could be a suitable application for large-scale EM to investigate other cellular interactions.

ACKNOWLEDGMENTS:

This research was supported by KBRI basic research program through Korea Brain Research Institute funded by Ministry of Science and ICT (19-BR-01-08), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1A6A3A11035628). SCO1-APEX2 and HRP-KDEL plasmids were kindly provided by Hyun-Woo Rhee (Seoul National University). TEM data were acquired at Brain Research Core Facilities in KBRI.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Krols, M. et al. Mitochondria-associated membranes as hubs for neurodegeneration. *Acta Neuropathologica.* **131** (4), 505-523 (2016).

2. Svendsen, E. J., Pedersen, R., Moen, A., Bjork, I. T. Exploring perspectives on restraint during

441 medical procedures in paediatric care: a qualitative interview study with nurses and physicians.
442 *International Journal of Qualitative Studies on Health and Well-being.* **12** (1), 1363623 (2017).
443 3. Shim, S. H. et al. Super-resolution fluorescence imaging of organelles in live cells with
444 photoswitchable membrane probes. *Proceedings of the National Academy of Sciences of the*
445 *United States of America.* **109** (35), 13978-13983 (2012).
446 4. Csordas, G. et al. Structural and functional features and significance of the physical linkage
447 between ER and mitochondria. *The Journal of Cell Biology.* **174** (7), 915-921 (2006).
448 5. Kremer, A. et al. Developing 3D SEM in a broad biological context. *Journal of Microscopy.* **259**
449 (2), 80-96 (2015).
450 6. Titze, B., Genoud, C. Volume scanning electron microscopy for imaging biological
451 ultrastructure. *Biology of the Cell.* **108** (11), 307-323 (2016).
452 7. Burel, A. et al. A targeted 3D EM and correlative microscopy method using SEM array
453 tomography. *Development.* **145** (12), (2018).
454 8. Micheva, K. D., Smith, S. J. Array tomography: a new tool for imaging the molecular
455 architecture and ultrastructure of neural circuits. *Neuron.* **55** (1), 25-36 (2007).
456 9. Seligman, A. M., Wasserkrug, H. L., Hanker, J. S. A new staining method (OTO) for enhancing
457 contrast of lipid--containing membranes and droplets in osmium tetroxide--fixed tissue with
458 osmiophilic thiocarbohydrazide(TCH). *The Journal of Cell Biology.* **30** (2), 424-432 (1966).
459 10. Lee, S. Y. et al. APEX Fingerprinting Reveals the Subcellular Localization of Proteins of Interest.
460 *Cell Reports.* **15** (8), 1837-1847 (2016).
461 11. Lam, S. S. et al. Directed evolution of APEX2 for electron microscopy and proximity labeling.
462 *Nature Methods.* **12** (1), 51-54 (2015).
463 12. Schikorski, T., Young, S. M., Jr., Hu, Y. Horseradish peroxidase cDNA as a marker for electron
464 microscopy in neurons. *Journal of Neuroscience Methods.* **165** (2), 210-215 (2007).
465 13. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nature*
466 *Methods.* **9** (7), 676-682 (2012).
467 14. Cardona, A. et al. TrakEM2 software for neural circuit reconstruction. *PLOS ONE.* **7** (6), e38011
468 (2012).
469 15. Kremer, J. R., Mastronarde, D. N., McIntosh, J. R. Computer visualization of three-dimensional
470 image data using IMOD. *Journal of Structural Biology.* **116** (1), 71-76 (1996).
471 16. Shu, X. et al. A genetically encoded tag for correlated light and electron microscopy of intact
472 cells, tissues, and organisms. *PLOS Biology.* **9** (4), e1001041 (2011).
473 17. Kijanka, M. et al. A novel immuno-gold labeling protocol for nanobody-based detection of
474 HER2 in breast cancer cells using immuno-electron microscopy. *Journal of Structural Biology.* **199**
475 (1), 1-11 (2017).
476 18. Ariotti, N., Hall, T. E., Parton, R. G. Correlative light and electron microscopic detection of GFP-
477 labeled proteins using modular APEX. *Methods in Cell Biology.* **140**, 105-121 (2017).
478 19. Hua, Y., Laserstein, P., Helmstaedter, M. Large-volume en-bloc staining for electron
479 microscopy-based connectomics. *Nature Communications.* **6**, 7923 (2015).
480 20. Shu, X. et al. A Genetically Encoded Tag for Correlated Light and Electron Microscopy of Intact
481 Cells, Tissues, and Organisms. *PLOS Biology.* **9** (4), e1001041 (2011).
482 21. Horstmann, H., Vasileva, M., Kuner, T. Photooxidation-Guided Ultrastructural Identification
483 and Analysis of Cells in Neuronal Tissue Labeled with Green Fluorescent Protein. *PLOS ONE.* **8** (5),
484 e64764 (2013).

22. Martell, J. D., Deerinck, T. J., Lam, S. S., Ellisman, M. H., Ting, A. Y. Electron microscopy using the genetically encoded APEX2 tag in cultured mammalian cells. *Nature Protocols.* **12** (9), 1792-1816 (2017).
23. Lam, S. S. et al. Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nature Methods.* **12** (1), 51-54 (2015).
24. Shi, Y., Wang, L., Zhang, J., Zhai, Y., Sun, F. Determining the target protein localization in 3D using the combination of FIB-SEM and APEX2. *Biochemistry and Biophysics Reports.* **3** (4), 92-99 (2017).

Figure 1

1 day

Cell culture

2 day

Plasmid transfection

Chemical Fixation

3 day

DAB labeling reaction by APEX2

Light Microscopic observation
(Record the cell location)

High – contrast
en bloc heavy metal staining

4,5 day

Dehydration

Embedding

Serial section by Ultra-microtome

6,7 day

Coated with carbon

TEM or SEM observation

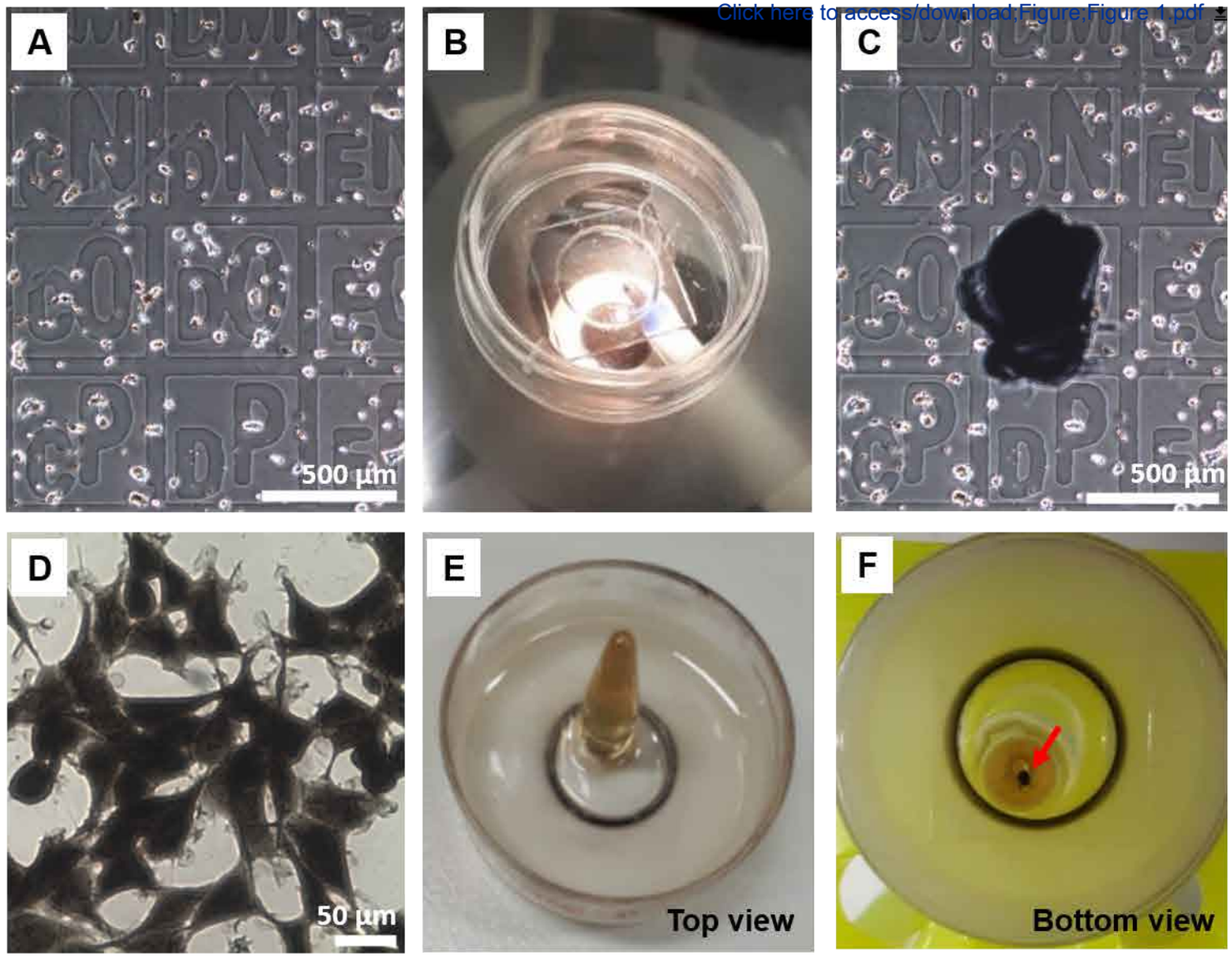


Figure 2

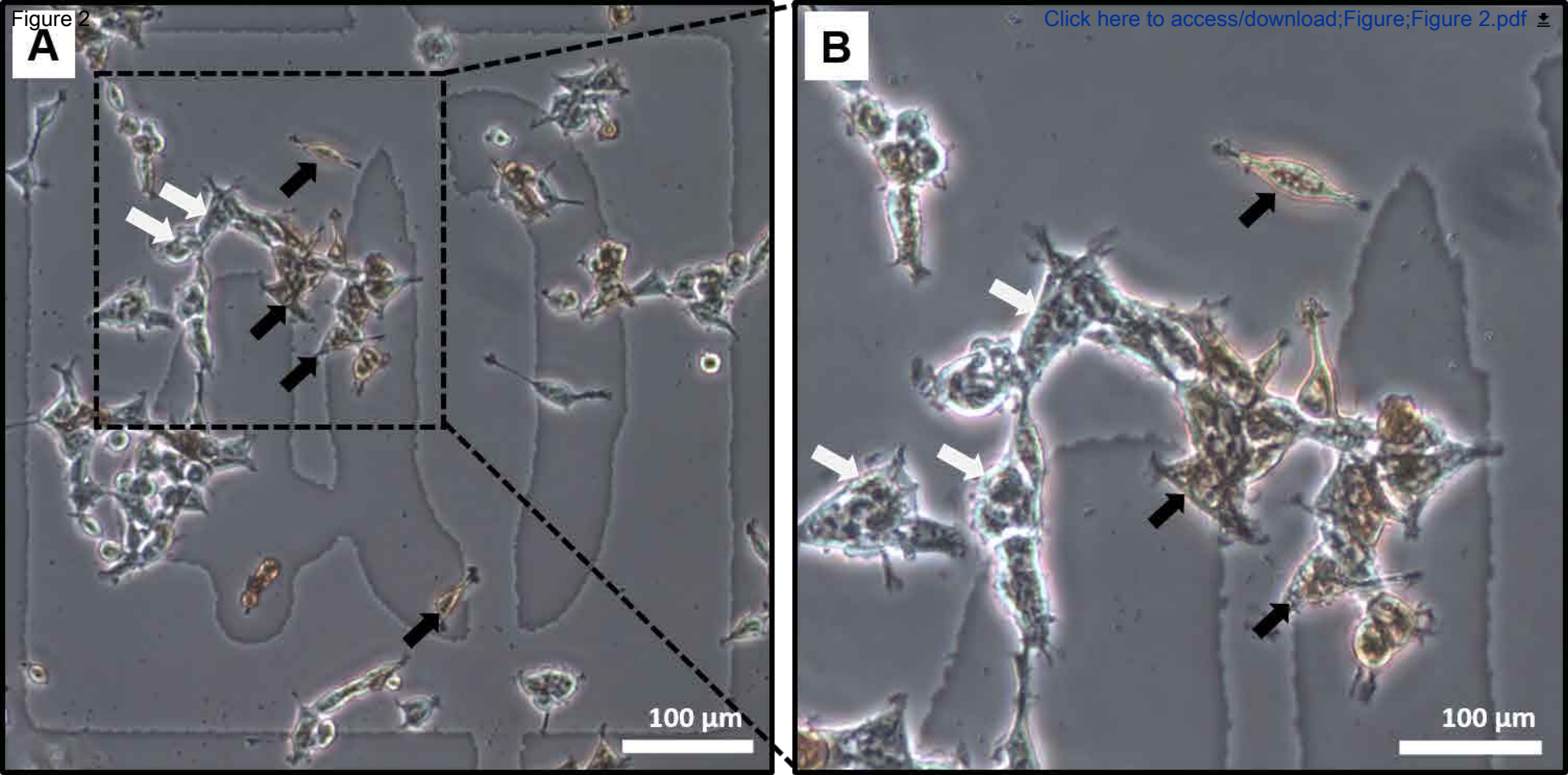
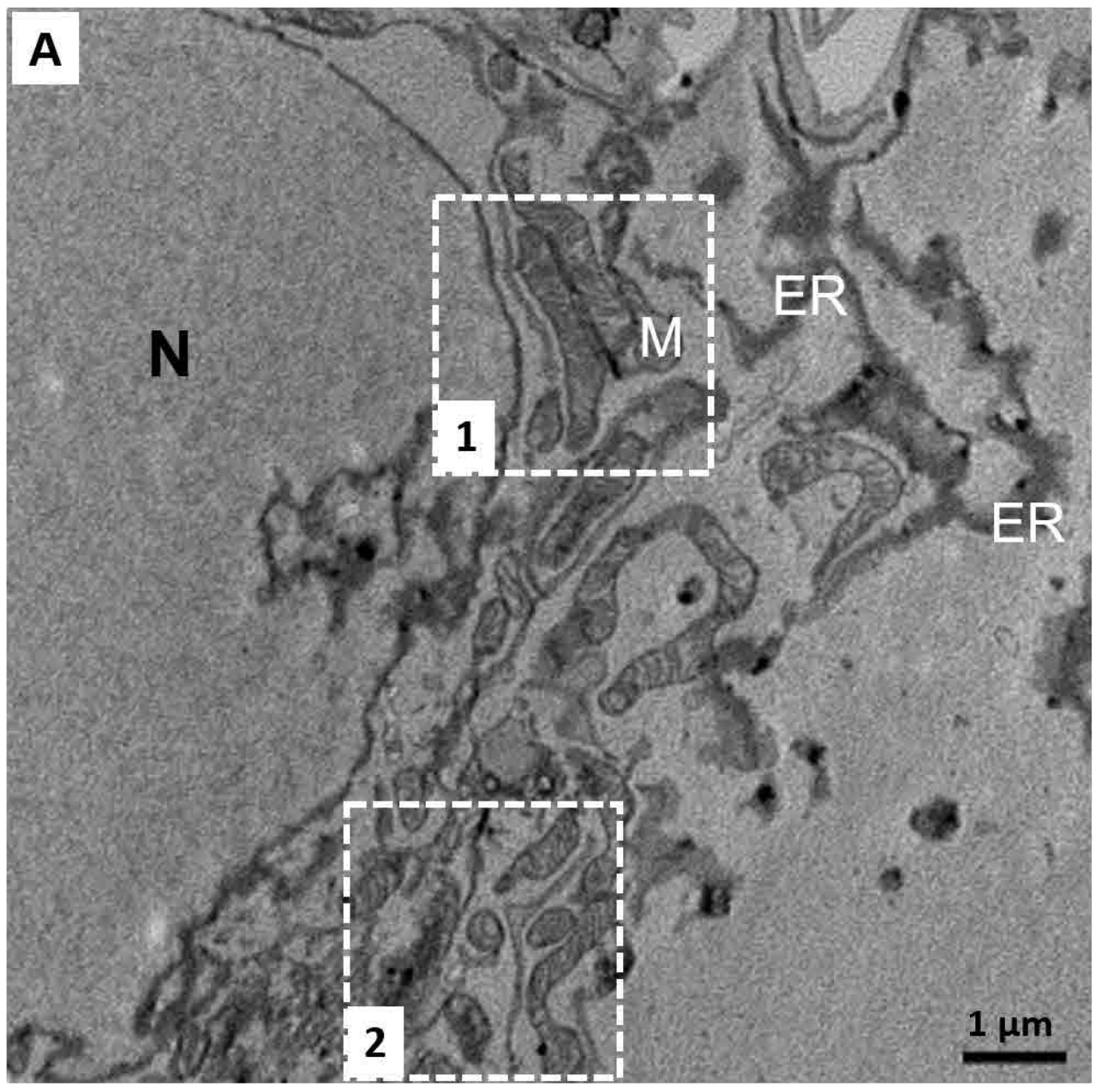
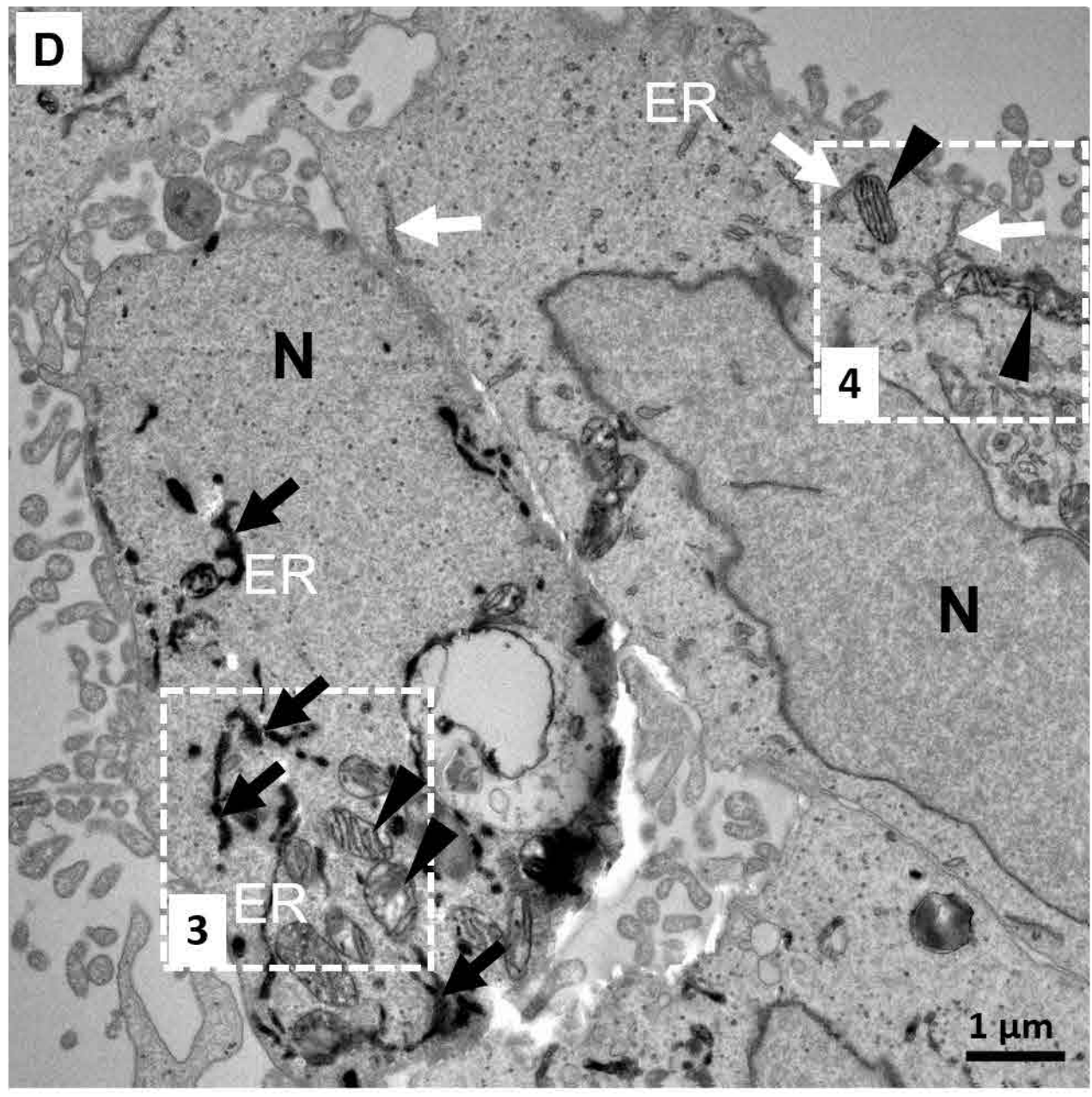


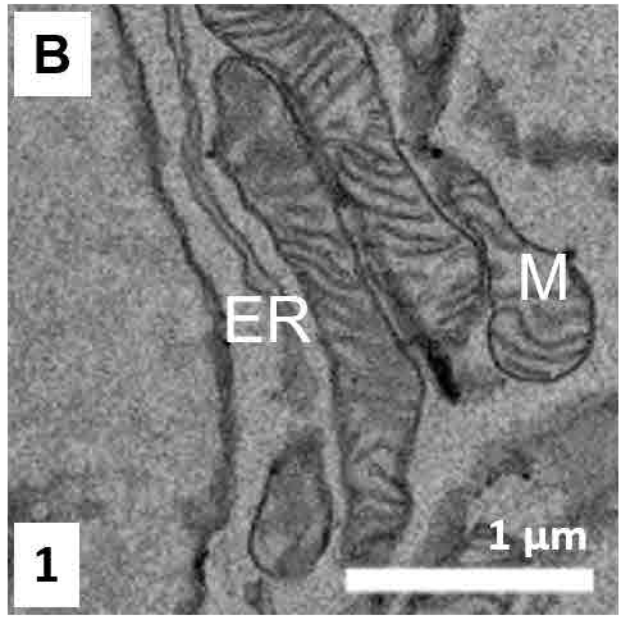
Figure 3

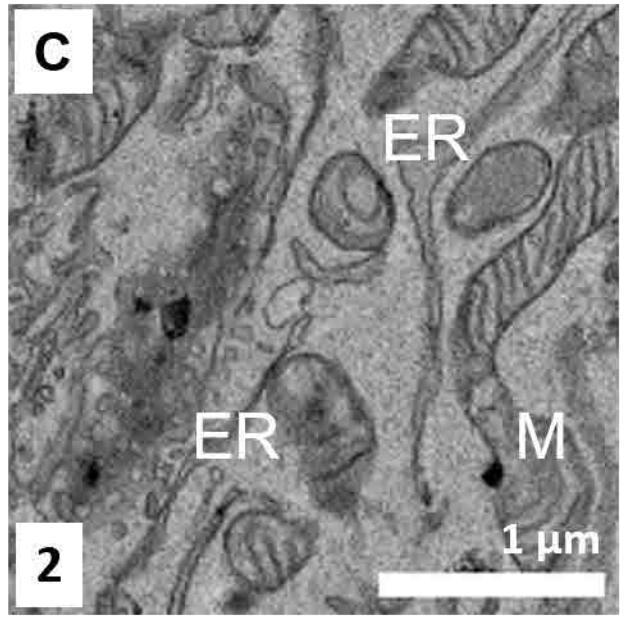
Untransfected cell

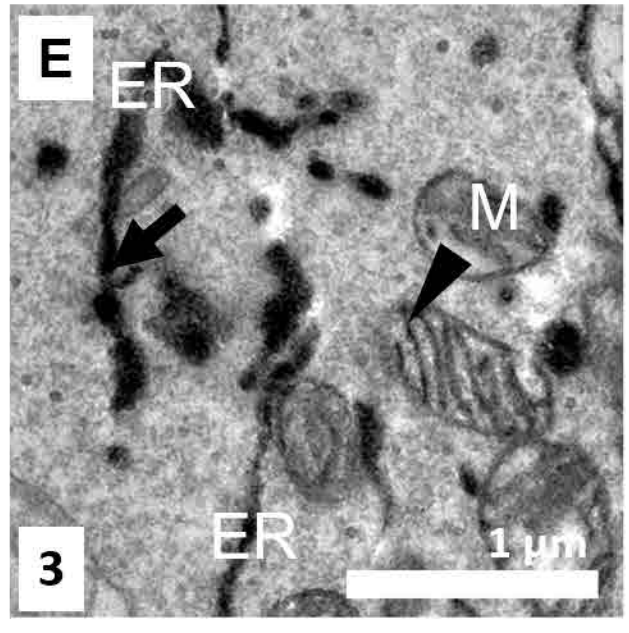
SCO1-APEX2 + HRP-KDEL

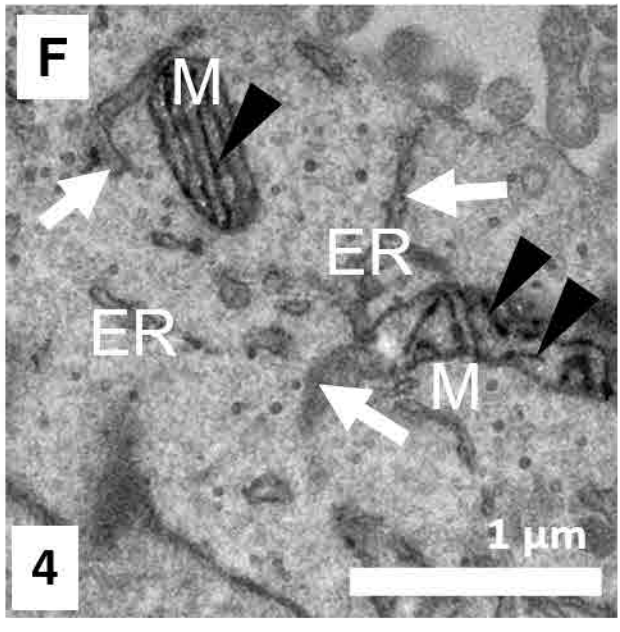




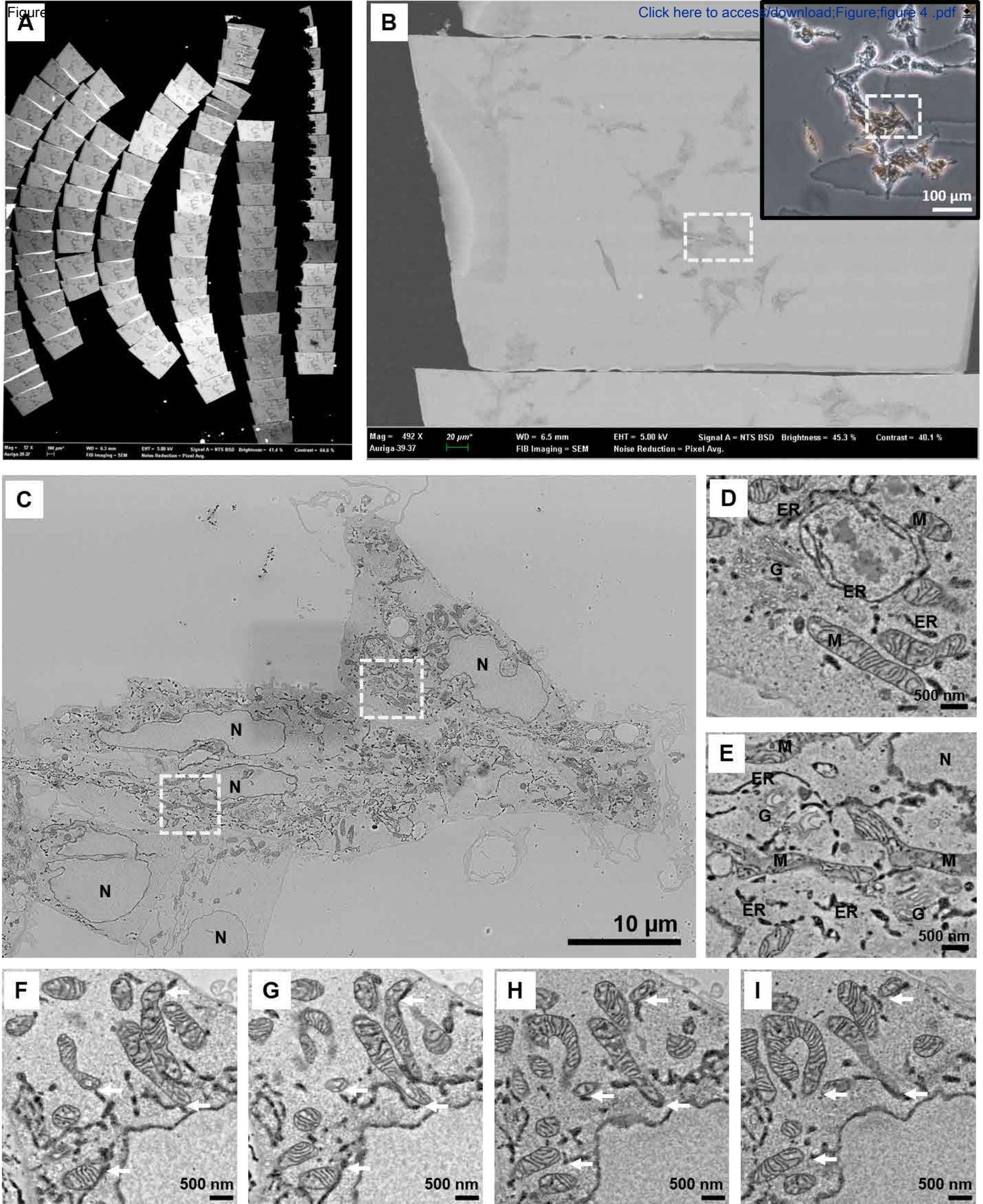








Figure



supple 1

Click here to access/download

Video or Animated Figure

Supple S1_stack.mp4

supple 2

Click here to access/download

Video or Animated Figure

Supple S2_crop.avi

supple 3

Click here to access/download

Video or Animated Figure

Supple S3_3D.avi

Table 1

Click here to access/download;Table;Table1_of_Solution recipes.xlsx

Solutions / mixtures	Concentration
Sodium cacodylate solution	0.3 M
	0.1 M
Cell fixation solution	-
Glycine solution	0.02 M
DAB solution	10x
	1x
Potassium ferrocyanide	3% (wt/vol)
Reduced osmium tetroxide solution	2% (vol/vol)
Aqueous uranyl acetate	1% (wt/vol)
Thiocarbohydrazide (TCH) solution	-
Aspartic acid solution	0.03 M
Walton's lead aspartate solution	-

Solution recipe
Dissolve 12.84 g of cacodylic acid in 160 mL of distilled water. Adjust pH to 7.4 with 0.1 M HCl then make up to 200 mL with distilled water.
Dilute the 3x sodium cacodylate solution threefold using distilled water.
1% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate solution (pH 7.0).
Dissolve 75 mg of glycine in 50 mL of 1x sodium cacodylate buffer.
Total 50 mg of DAB powder dissolve in 10 mL of 0.1 M HCl solution at the room temperature with extensive vortexing for 10−20 min. Undissolved material is removed by centrifuge at 13,523 x *g* at room temperature for 10 min. Small volume aliquots (e.g., 1 mL) of 10x DAB solution and store them at - 80 °C for several months.
Thaw a 1 mL aliquot of 10x DAB, and then mix it with 3.33 mL of cold 0.3 M sodium cacodylate solution and 5.67 mL of cold water, finally add 10 µL of 30% H_2O_2.
Dissolve 1.5 g of tetrapotassium ferrocyanide in 50 mL of 0.2 M sodium cacodylate solution.
3% potassium ferrocyanide in 0.2 M sodium cacodylate solution is combined with equal volume of 4% aqueous osmium tetroxide.
Dissolve 1 g of uranyl acetate in 90 mL of distilled water in RT. Almost all the uranyl acetate has dissolved, then make up to 100 mL with distilled water.
Add 0.1 g of thiocarbohydrazide to 10 mL of distilled water and dissolve in a 60 °C oven for 1 h.
Add 0.2 g of L-aspartic acid to 50 mL of distilled water.
Dissolve 0.066 g of lead nitrate in 10 mL of 0.03 M aspartic acid solution and adjust pH 5.5 with 1 N KOH.

Name of Material/Equipment	Company	Catalog Number
Glutaraldehyde	EMS	16200
Paraformaldehyde	EMS	19210
Sodium cacodylate	EMS	12300
Osmium tetroxide 4 % aqueous solution	EMS	16320
Epon 812	EMS	14120
Ultra-microtome Leica ARTOS 3D	Leica	ARTOS 3D
Uranyl acetate	EMS	22400
Lead citrate	EMS	17900
35mm Gridded coverslip dish	Mattek	P35G-1.5-14-CGRD
Glow discharger	Pelco	easiGlow
Formvar carbon coated Copper Grid	Ted Pella	01805-F
Hydrochloric acid	SIGMA	258148
Fugene HD	Promega	E2311
Glycine	SIGMA	G8898
3,3′ -diaminobenzamidine (DAB)	SIGMA	D8001
30% Hydrogen peroxide solution	Merck	107210
Potassium hexacyanoferrate(II) trihydrate	SIGMA	P3289
0.22 um syringe filter	Sartorius	16534
Thiocarbonyldihydrazide	SIGMA	223220
Potassium hydroxide	Fluka	10193426
L-aspartic acid	SIGMA	A9256
Ethanol	Merck	100983
Transmission electron microscopy	FEI	Tecnai G2
Indium tin oxide (ITO) coated glass coverslips	SPI	06489-AB
Isopropanol	Fisher Bioreagents	BP2618-1
Diamond knife	Leica	AT-4
Inveted light microscopy	Nikon	ECLipse TS100
Scanning electron microscopy	Zeiss	Auriga

Comments/Description
Use only in fume hood
Use only in fume hood
Use only in fume hood
EMbed 812- 20 ml/ DDSA- 16 ml/ NMA- 8 ml/ DMP-30 - 0.8 ml
Hazardous chemical
Hazardous chemical
Use only in fume hood
fragil glass

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Mitochondria and endoplasmic reticulum Imaging by correlative right and volume ele on microscop

Author(s): Minkyo Jung and Ji Young Mun

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Ji Young Mun
Department:	Department of structure and function of neural network
Institution:	Korea brain Research Institute
Title:	Ph.D
Signature:	[signature]
Date:	1/30/19

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear editor,

Thank you for comments. Hopefully we have addressed all concerns to your satisfaction.

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

➔ We edited the style.

2. Please address the following comments from reviewers:
a. What is the success rate per preparation and what are your criteria for success? This is important as this is being advertised as a method for not a "good facility" but an average program and will involve a rather serious commitment by the reader and realistic expected outcomes need to be reported.

➔ The success rate is same as cell transfection rate. But the beauty of this technique is we can select transfected cells in light microscopy before electron microscopy. Because of it, we don't need to spend a lot of time to find out the cells with proteins we added.

b. The authors need to discuss broader applications of this protocol. Can this protocol be applied to other cultured cell lines? Even more interestingly, can it be used in primary cultures? I am also wondering if this protocol could be modified to visualize different organelles? If so, it would be very useful if the authors can create a table describing genetically encoded protein tags that could be used to label other organelles. (Editor: Please incorporate the authors' response into the discussion section).

➔ We tested this technique in different cell including Hela, HEK293T, and primary neuron cell. Even though we applied this technique for mitochondria and ER, Martell et al showed plasma membrane, nucleus, tubulin, actin (PMID :28796234) tagged with APEX in COS 7 and HEK293T cell. We added a sentence in discussion section.

3. Please address specific comments marked in the attached manuscript.

➔ We edited it.

4. Figures: Please do not italicize the micro symbol "μ". We edited it.

5. Figure 3A,D: Please use "μ" instead of "u" for the micro symbol.

➔ We edited it.

6. Figure 4: Please include a space between the number and its unit of the scale bar (i.e., 500 nm, etc.). We edited it.
7. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file. Please remove the titles from the uploaded figures.