



Correlative Light and Electron Microscopy for Nanoparticle–Cell Interaction and Protein Localization

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Introduction

Correlative light and electron microscopy (CLEM) is a method where a specimen (i.e., cell) is observed using light (or fluorescent) microscopy and electron microscopy (EM), after which the correlation between the light microscopy and electron microscopy images is determined (Fig. 1) (Abandowitz and Geissinger 1975; Rieder and Bowser 1985; Webster et al. 1978). Light microscopy is one of the tools used to observe dynamic biological phenomena, including molecules within a cell. Various fluorescence dyes and fluorescence proteins (FPs) have been used to examine sub-cellular localization and co-localization of target proteins in living cells. Light microscopy has a key limitation in the form of spatial resolution, mainly due to

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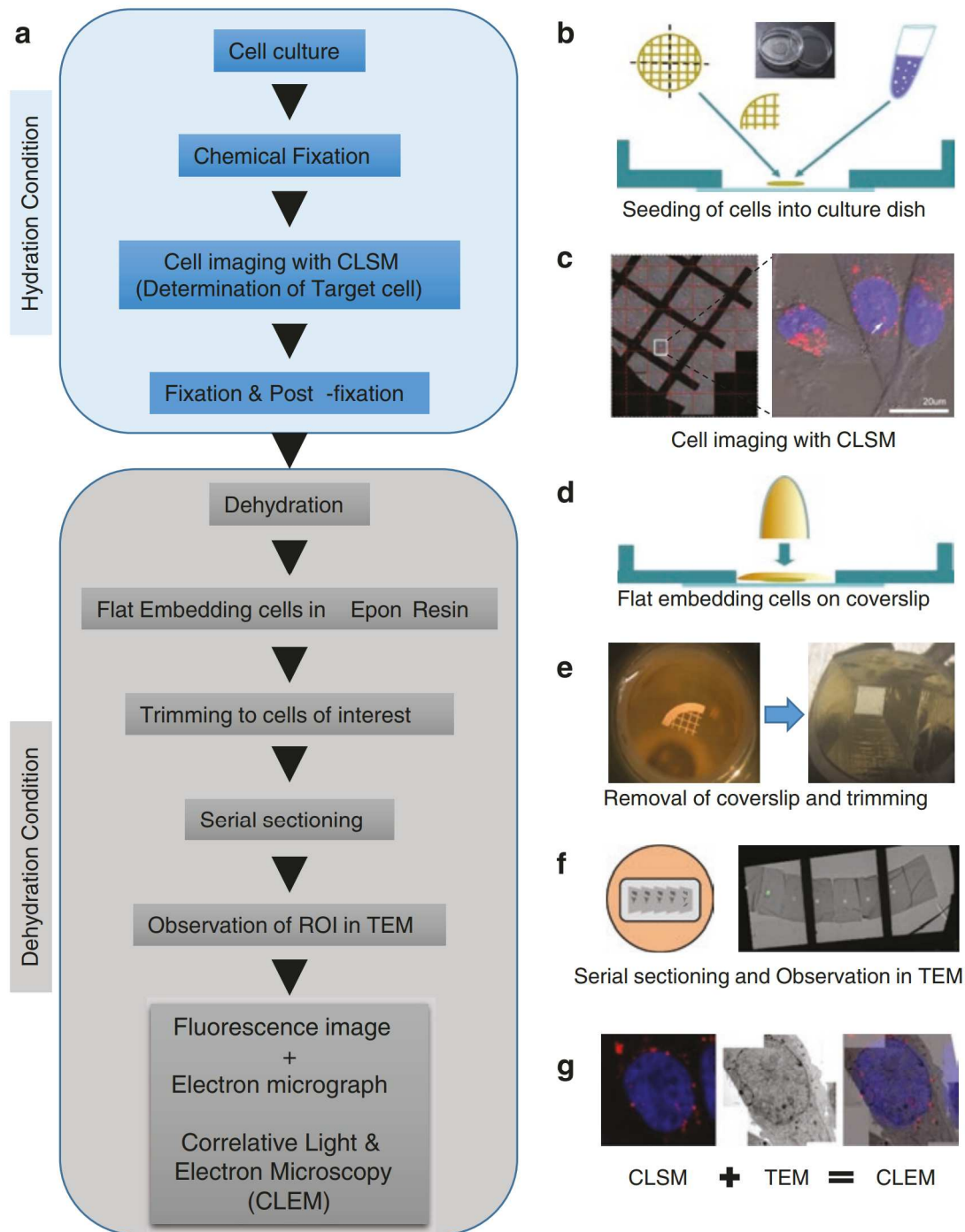


Fig. 1 Workflow of correlative light and transmission electron microscopy. **(a)** Flowchart of the major steps in correlative light and transmission electron microscopy imaging. **(b)** Plastic culture dish with a coverslip attached to the bottom of the dish. The gold grid is cut into pieces and placed at the bottom of the glass. Mammalian cells were spread on the cut gold grid. **(c)** Observation of a HeLa cell. To identify the target cells, a whole grid mesh was scanned using the tile scan function. Three-dimensional fluorescence images of the cell obtained through confocal microscopy using a high-performance lens at intervals of 250 nm in the optical Z-section. **(d)** Flat embedding in Spurr resin. **(e)** Using the transferred gold grid mesh, the block was trimmed so that cells in the area of interest and then serial sections were cut using an ultramicrotome. **(f)** Serial sections were mounted on a formvar carbon-coated single-slot grid or three-hole grid. **(g)** Correlate fluorescent images with electron microscopy (EM) images

optical diffraction, so that fluorescence microscopy (i.e., confocal laser scanning microscopy) can only provide images with relatively low resolution, over approximately 200 nm.

Newly developed super-resolution light microscopy techniques can obtain high spatial resolution in the 20–100 nm range (Sahl et al. 2017). Electron microscopy provides sub-nanometer scale resolution images using resin-embedded biological samples. However, it cannot reveal highly dynamic phenomena within living cells. Therefore, the correlation between light microscopy and electron microscopy in a sample (i.e., single cells) presents dual advantages in the form of dynamic fluorescent imaging of live cells and the high resolutions associated with electron microscopy imaging. The advanced technique has been employed in various biological studies, such as virus–cell interactions, combining live-cell imaging, and immunolabeling of ultrathin cryosections, and cellular localization of quantum dot nanoparticles (Bykov et al. 2016; Han et al. 2019; van Rijnsoever et al. 2008).

The use of tagged FPs and various fluorescent nanoparticle probes has allowed not only the dynamic movement of intracellular organelles but also specific diffusional and interactional properties of the fluorescent proteins to be tracked using confocal microscopy and confocal-related analytical techniques, such as fluorescence recovery after photobleaching (FRAP), fluorescence resonance energy transfer (FRET), fluorescence correlation spectroscopy (FCS), and fluorescence cross-correlation spectroscopy (Ishikawa-Ankerhold et al. 2012; Pack et al. 2012, 2014a, b). Notably, a CLEM method that uses ascorbate peroxidase (APEX) tags to visualize GFP-tagged proteins has been developed over the past few years (Martell et al. 2012, 2017), and it can be combined with the fluorescent techniques described above. Since cells traced using such fluorescent methods could be fixed at the moment of interest and analyzed using CLEM, a lot of information on both the cellular behavior of GFP-tagged target molecules and detailed structural status of cellular microenvironments surrounding the target molecules is available, which is beneficial particularly in the fields of nanomedicine and drug delivery (Jin et al. 2016; Patra et al. 2018). In addition, various clinical anticancer drugs and nanomaterials targeting cells can be fluorescently labeled and then exploited using CLEM and the various fluorescent methods (Darcy et al. 2006). For example, label-free or fluorescently labeled Herceptin, one of the famous anticancer drugs targeting Her2 receptors on cancer cells, strongly interacts with the cell surface through GFP-tagged Her2 receptors (refer to Chap. 1 in this book). Consequently, fluorescently labeled drugs, drug carriers such as nanoparticles, and GFP-tagged proteins are all good candidates for CLEM applications.

Recently, fluorescent NPs have emerged as promising and biocompatible imaging probes in confocal microscopy and have been used as drug carriers for biomedicine and nanomedicine applications. In practice, numerous studies have demonstrated the application of nanomaterials as drug carriers (Kulkarni et al. 2010; Radu et al. 2004; Regier et al. 2012). Among various nanoparticles, silica nanoparticles (Si-NPs) are the most commonly applied nanomaterials in such applications due to their easy surface modification, low cell toxicity, and high levels of uptake into eukaryotic cells (Radu et al. 2004; Sailor and Park 2012). In addition, silica-based

fluorescent Si-NPs have been employed as analytical probes for the study of cellular distribution and diffusional dynamics due to their strong fluorescent signals, biological inertness (i.e., low cytotoxicity), and high solubility (Pack et al. 2012). A previous study based on confocal imaging and FCS analysis demonstrated the existence of various types of Si-FNP cellular diffusional movements in peptide-coated and non-coated Si-FNPs. However, the reason for the slowed diffusion of the coated Si-FNPs and the potential interactions between NPs and various cellular organelles were not investigated.

In this chapter, we introduce a straightforward imaging approach using CLEM for the identification of the exact localizations of Si-FMNPs or APEX2 conjugated specific proteins in the cellular compartments, such as the cytosol, nucleus, and outer surface of the plasma membrane at the single-particle level.

Human epithelial cervical carcinoma (HeLa) cells electroporated with Si-FMNP have been imaged using three-dimensional (3D) confocal microscopy and examined under transmission electron microscopy (TEM) to observe the intracellular distribution of NPs. Conversely, cells were incubated after treatment with culture media with NPs, and the distribution of Si-FMNPs on the cell surface was imaged under confocal microscopy and scanning electron microscopy (SEM) (Fig. 2).

Material and Methods

Materials

1. Cell culture dish: Gridded glass-bottom cell culture dishes (35-mm petri dish, No. 1.5 cover glass, P35G-1.5-14-CGRD, MatTek).
2. Silica nanoparticles containing magnetic core (NEO-STEM™ Si-FMNP containing RITC dye, Biterials Co., Ltd., Korea).
3. Glutaraldehyde, 25% in water, electron microscopy grade (Electron Microscopy Sciences, 16200) (see Note 1).
4. 16% Paraformaldehyde (Electron Microscopy Sciences, 15700) (see Note 1).
5. Osmium tetroxide, 4% water solution (Electron Microscopy Sciences, 16320) (see Note 1).
6. Uranyl acetate (Electron Microscopy Sciences, 22400).
7. A graded series of ethanol solutions: 50%, 60%, 70%, 80%, 90%, 95%, 100% (v/v), ethanol (Merck, 100983) in distilled water.
8. Embedding resin (Electron Microscopy Sciences, 14120).
9. Sodium cacodylate buffer, 0.1 M, pH 7.4 (sodium cacodylate trihydrate, Electron Microscopy Sciences, 12300): For 0.1 M sodium cacodylate, dissolve 4.28 g 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.
10. Lead citrate (Electron Microscopy Sciences, 17800).
11. Single-slot copper grids (Ted Pella, 01805-F).
12. For a 10× DAB solution, dissolve 50 mg of DAB powder (3,3'-diaminobenzamide, Sigma-Aldrich, D8001) in 10 mL of 0.1 M HCl solution at room temperature with strong vortexing for 10–20 min. Undissolved material is removed by centrifuging at 13,523×g at room temperature for 10 min.

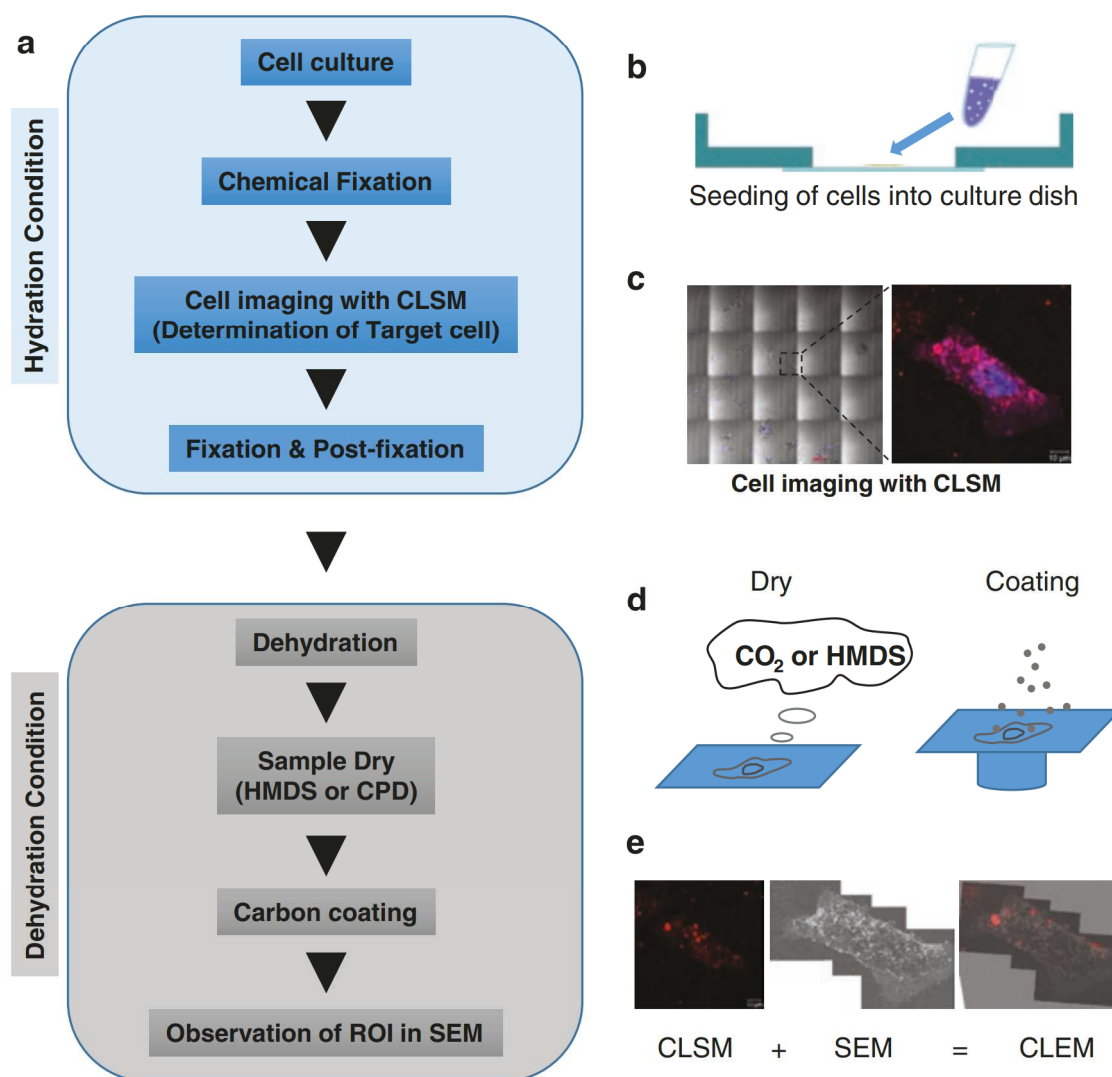


Fig. 2 Workflow of correlative light and scanning electron microscopy (SEM). **(a)** Flowchart of the major steps in the correlative light and scanning electron microscopy imaging procedure. **(b)** Plastic culture dish with a gridded coverslip attached to the bottom of the dish. Mammalian cells were spread on the bottom of the gridded glass coverslip. **(c)** To identify the target cells, a large area was scanned using the tile scan function. High-resolution fluorescence images of the cell were obtained through confocal microscopy using a high-performance lens. **(d)** Specimens were dried using a critical point drier or hexamethyldisilazane (HMDS) and then coated with carbon or metal. **(e)** Correlated fluorescent and SEM images

13. For a 1× DAB solution, 0.3 M cacodylate solution 3.33 mL + 30% H_2O_2 10 μL + 5.67 mL of cold water +1 mL 10× DAB solution.
14. 30% Hydrogen peroxide solution (Merck, 107210).
15. Thiocarbonyldihydrazide (TCH, Sigma, 223220) (see Note 1).
16. Hoechst 33342 (NucBlue Live Ready Probes, Thermo Fisher, R37605).
17. Hexamethyldisilazane (HMDS, Sigma 440191).
18. CellMask Deep Red (Thermo Fisher, C10046).

TEM-Based CLEM Methods

1. Cell culture and treatment of nanoparticles (or transfection).
 - 1.1. HeLa cells are grown in a humidified atmosphere containing 5% CO₂ at 37 °C in Dulbecco's Modified Eagle Medium (DMEM; Gibco Canada Ltd., Burlington, Canada) supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin.
 - 1.2. Cut the EM gold mesh grid to one-fourth size (or whole grid) and sterilize it with 70% alcohol, then place it in a 35-mm glass-bottom culture dish (see Note 2).
 - 1.3. Seed 1×10^5 cells by placing them onto the EM gold mesh grid in the culture dish. It is important to maintain the cells at low confluence in the mesh grid (see Note 3).
 - 1.4. The day after seeding the cells, to introduce the cellular uptake of Si-FMNP through endocytosis, treat with a Si-FMNP solution (0.2 mg/mL) for 24 h on a culture dish.
 - 1.5. After 24 h, the cells are counterstained with Hoechst 33342 (0.5 µg/ml).
2. Fixation.
 - 2.1. To fix the cells, remove all the culture media and wash the cells briefly with pre-warmed PBS and then immediately add 1.5 ml of fixation solution (1% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate solution [pH 7.0]) by gentle pipetting at room temperature (see Note 1).
 - 2.2. After fixing, wash the cells three times, for 10 min each time, with cold 0.1 M sodium cacodylate buffer at room temperature.
3. Fluorescence (or confocal) microscopy.
 - 3.1. Observe the overall location of the target cells in the mesh grid using a 40× lens on the confocal microscope. Each single image shot in tile scan mode using the motorizing stage produces a single large image (Fig. 4a). This overview image serves as a map for the detection of specific target cells in the plastic-embedded samples.
 - 3.2. The target cells that incorporate the nanoparticles are imaged using high-magnification images (Fig. 4b) and z-stack images (Fig. 4c).
4. TEM sample preparation and flat embedding in epoxy resin.
 - 4.1. Post-fix the cells in 2% (w/v) osmium tetroxide (O₈O₄) and 1.5% (w/v) potassium ferrocyanide in 0.1 M sodium cacodylate buffer for 1 h at room temperature.
 - 4.2. Remove the fixative and wash the cells three times, for 10 min each time, with distilled water at room temperature.
 - 4.3. Add 1 ml of 1% uranyl acetate (aqueous), and leave the cells in cold conditions (4 °C).
 - 4.4. On the following day, wash the cells three times, for 10 min each time, with distilled water at room temperature.
 - 4.5. Dehydrate the samples in a graded series of 2 mL ethanol solutions, 50%, 60%, 70%, 80%, 90%, 95%, and 100% (w/v), for 20 min each at room temperature.
 - 4.6. Remove the ethanol and incubate for 1 h in a 25% (v/v) epoxy resin ethanol mixture at room temperature.

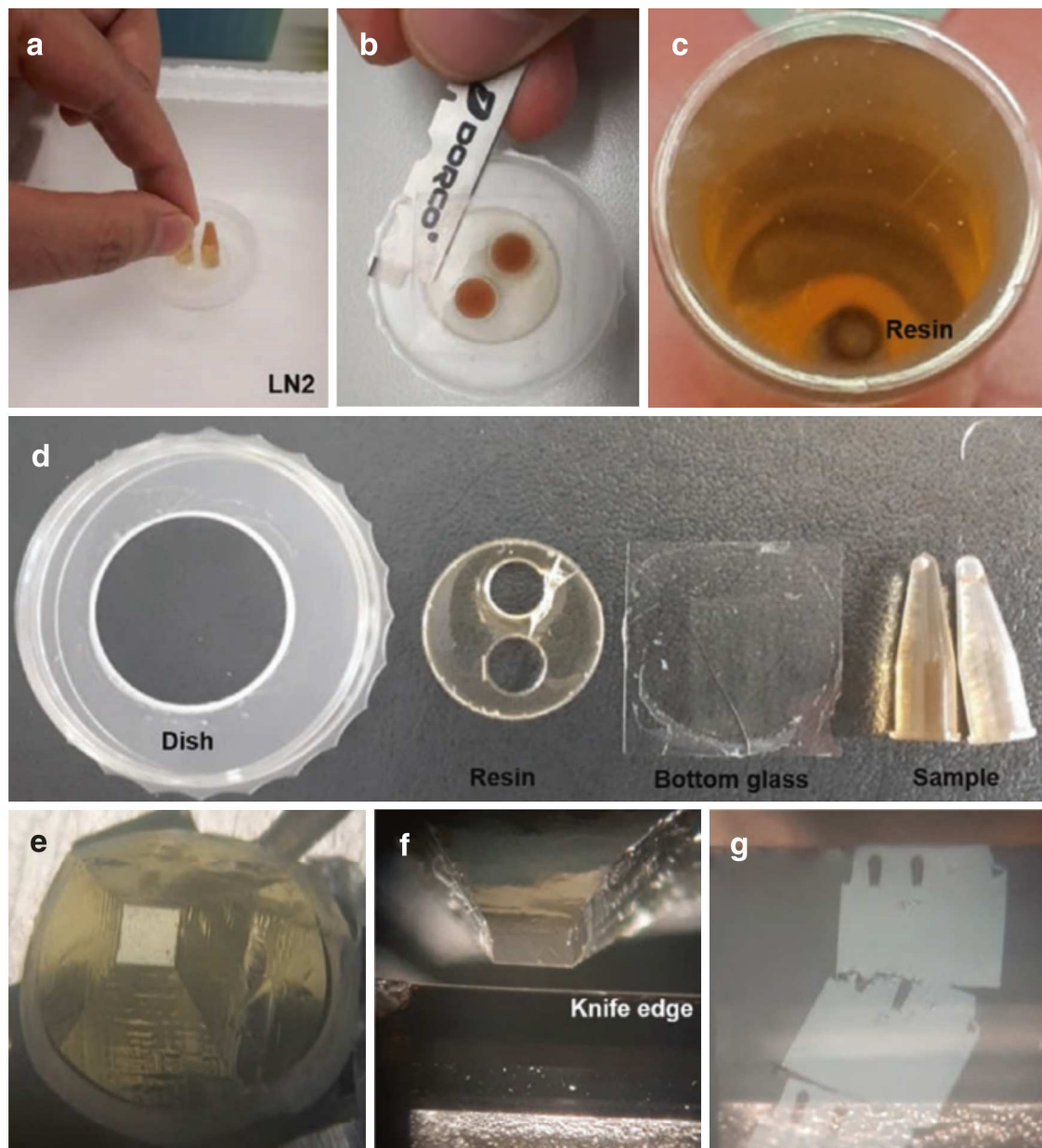


Fig. 3 Removal of the bottom glass and dish from the epoxy resin and serial sectioning. (a) Embedded capsules are removed from the glass bottom coverslip by dipping them into liquid nitrogen. (b) Using a razor to separate the bottom glass from the plastic dish. (c) A resin block well separated from the bottom glass. (d) Well-separated bottom glass from the dish and samples are shown. (e) Trimming of the four sides of the target position using a razor blade. (f) Alignment of the block-face parallel to the knife edge. (g) Serial sectioned ribbons

- 4.7. Remove the medium and incubate for 1 h in a 50% (v/v) epoxy resin ethanol mixture at room temperature.
- 4.8. Remove the medium and incubate for 1 h in a 75% (v/v) epoxy resin ethanol mixture at room temperature.
- 4.9. Remove the medium and incubate in a 100% low-viscosity embedding medium mixture overnight at room temperature.
- 4.10. On the following day, finally, incubate in a 100% low-viscosity embedding medium mixture for 3 h at room temperature.

- 4.11. Fill a PCR tube or a BEEM capsule with 100% fresh epoxy resin, and place the tube or capsule upside down on the gold grid and incubate for 24 h at 60 °C.
5. Trimming of the sample block and serial sectioning.
 - 5.1. After the complete polymerization of the epoxy resin, the bottom glass is removed from the epoxy resin by briefly dipping the dish in liquid nitrogen (Fig. 3a–d).
 - 5.2. Trim the resin block around the target position using a razor blade. The shape of the block-face is made appropriate for rectangular cuts (Fig. 3e).
 - 5.3. Insert the sample block in the sample holder and set on a diamond knife. Align the leading and trailing edge of the sample block exactly parallel to the diamond knife edge (Fig. 3f).
 - 5.4. Cut about 50 nm of a thin section using an ultramicrotome. If the perfectly shaped block-face is not cut, the target cell already exists in the first section (Fig. 3g).
 - 5.5. Carefully collect the sections on the formvar carbon-coated slot grid and let them dry out.
6. Heavy metal staining.
 - 6.1. For electron microscopy observations, stain the grid with 2% (v/v) aqueous uranyl acetate for 20 min at room temperature.
 - 6.2. Wash the grid with drops (2 mL) of distilled water.
 - 6.3. Stain the grid with lead (II) nitrate solution for 10 min at room temperature.
 - 6.4. Wash the grid with drops (2 mL) of distilled water, and dry the remaining water using filter paper.
7. Electron microscopy.
 - 7.1. Obtain images of the serial sections under TEM.
 - 7.2. Observe the cell of interest and the ultrastructure region of interest of a similar position under confocal microscopy image (Fig. 4a, b) in the EM. First, obtain low magnification of a single cell or a small group (3–4 cells) of cells in the EM micrograph and compare with fluorescent images (Fig. 5).

SEM-Based CLEM Methods

1. Cell culture and treatment of nanoparticles.
 - 1.1. Culture HeLa cells in a humidified atmosphere containing 5% CO₂ at 37 °C in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin.
 - 1.2. Seed 1×10^5 cells by placing them onto a gridded glass-bottom dish. It is important to maintain the cells at low confluence in the gridded glass-bottom dishes.
 - 1.3. The day after seeding the cells, to trigger the cellular uptake of Si-FMNP through endocytosis, treat using a solution (0.2 mg/mL) of Si-FMNP for 12 h on a culture dish.

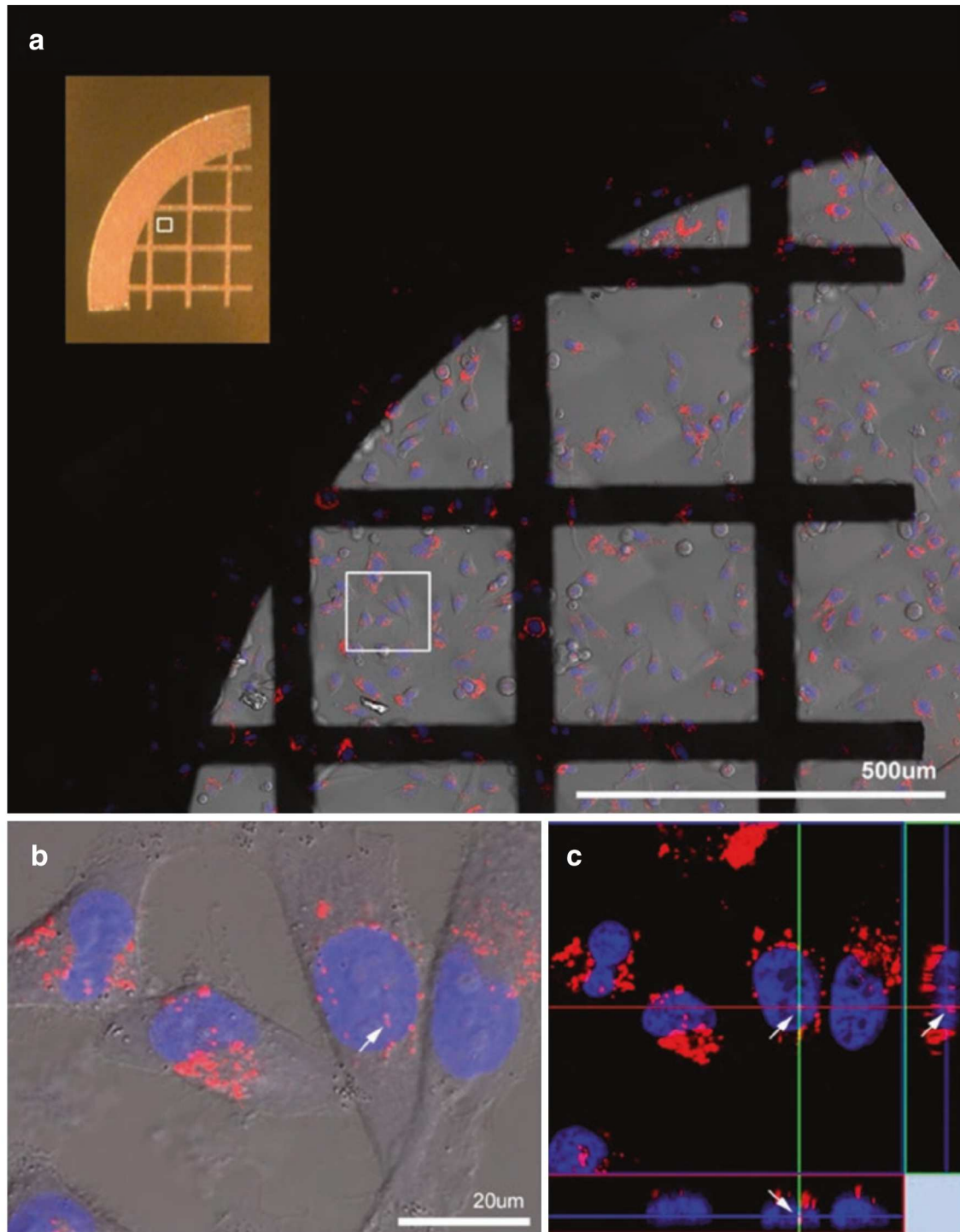


Fig. 4 Identification and selection of cells with silica NPs in the nuclei. Incorporation of Si-FMNPs into the cytosol and nuclei of living HeLa cells after electroporation of 0.2 mg/mL Si-FMNPs. Cells containing Si-FMNPs in the cytosol were imaged 24 h after electroporation. **(a)** A tiled image (9×11 stitched images with the total size of the grid) of the entire field of cells on the grid. The white box is a selected area with target cells. **(b)** A cell containing Si-FMNPs in the nucleus (white arrow) as well as in the cytosol was imaged 24 h after electroporation. **(c)** Orthogonal 3D image of the cell shown in B. The orthogonal Z-stack profile along the indicated lines in the x or y direction of the central xy image is shown in the rectangular windows below (x–z) and to the right (y–z) of the center (x–y) image. White arrows indicate aggregates of Si-FMNPs fixed in the nucleus. Red and blue fluorescence are originated from Si-FMNP and DAPI staining, respectively

2. Fixation and confocal light microscopy observation.
 - 2.1. To stain the plasma membrane and the nucleus, add 1 μ L of CellMask Deep Red and 300 nM DAPI stain solution to the culture media. Incubate for 10 min at 37 °C. Remove the staining solution and rinse the cells three times with pre-warmed PBS.
 - 2.2. To fix the cells, immediately add 1.5 ml of fixation solution (1% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate solution [pH 7.0]) by gentle pipetting for 20 min under dark conditions at 4 °C (see Note 2).
 - 2.3. After fixing, wash the cells three times, for 10 min each time, with cold 0.1 M sodium cacodylate buffer at room temperature.
 - 2.4. Observe the overall location of the target cells in the gridded glass-bottom dish by using a 40 $\times$ lens on the confocal microscope. Shoot each single image in the tile scan mode using the motorizing stage to produce a single large DIC image (Fig. 8a, b). The overview image serves as a map for the identification of specific target cells on the gridded bottom glass dish.
 - 2.5. Obtain high-magnification fluorescence images of the target cells that incorporate nanoparticles (Fig. 8c).
3. Sample preparation for SEM.
 - 3.1. Incubate cells for 20 min using a graded ethanol series (50%, 60%, 70%, 80%, 90%, 95%, and 100%) at room temperature.
 - 3.2. Remove the ethanol and incubate for 1 h in a 25% (v/v) HMDS ethanol mixture at room temperature (see Note 4).
 - 3.3. Remove the medium and incubate for 1 h in a 50% (v/v) HMDS ethanol mixture at room temperature.
 - 3.4. Remove the medium and incubate for 1 h in a 75% (v/v) HMDS ethanol mixture at room temperature.
 - 3.5. Remove the medium and incubate for 1 h in a 100% HMDS solution at room temperature.
 - 3.6. Air-dry the samples in dust-free conditions.
 - 3.7. Attach the bottom glass on aluminum stubs and then surface coat them using a 10-nm-thick platinum layer.
 - 3.8. Observe the glass surface under field emission SEM.

CLEM for the Identification of Specific Proteins Using Genetically Encoded Tag APEX2

Because the genetic probes do not require the permeability of the cell membrane to access the internal structure, they have the advantage of preserving the compact structure of the sample. In many previous studies, several gene probes, such as GFP (Horstmann et al. 2013), miniSOG (Shu et al. 2011), and APEX2 (engineered ascorbate peroxidase), have been reported using CLEM (Lam et al. 2015; Martell et al. 2017). GFP or miniSOG requires special light sources for DAB precipitation; however, APEX2 does not require special equipment. This section describes the procedures for preparing CLEM samples from cell monolayers.

1. Cell culture with patterned grid culture dish and cell transfection with plasmid vector.
 - 1.1. HeLa cells were cultured in a humidified atmosphere containing 5% CO₂ at 37 °C in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin.
 - 1.2. Seed 1×10^5 cells by placing them onto gridded glass-bottom dishes. It is important to maintain the cells at low confluence in the gridded glass-bottom dishes.
 - 1.3. On the day after seeding of the cells, introduce the Mito-V5-APEX2 plasmid to the cells with transfection reagent when the cells have grown to 50–60% confluence.
2. Light microscope observation of DAB staining for APEX2.
 - 2.1. 16–24 h after transfection, to fix the cells, remove all the culture media and wash the cells briefly with pre-warmed PBS. Subsequently, immediately add 1.5 ml of fixation solution (1% glutaraldehyde and 1% paraformaldehyde in 0.15 M sodium cacodylate solution [pH 7.0]) by gentle pipetting at room temperature (see Note 1).
 - 2.2. Wash fixed cells with cold 0.15 M sodium cacodylate buffer three times on ice.
 - 2.3. Add 1 ml of cold 50 mM glycine solution and incubate for 10 min on ice, and then wash three times, for 10 min each time, with cold 0.15 M sodium cacodylate buffer.
3. Prepare a fresh 1× DAB solution. Add 500 µL of 1× DAB solution and incubate on ice until a light brown stain is visible under a light microscope for 20 min.
4. Remove the DAB solution and wash three times, for 10 min each time, with cold 0.15 M sodium cacodylate buffer.
5. Observe using a phase-contrast microscope to record the location of the brown stained cells.
6. EM sample preparation and ultra-sections are similar to procedures described in 4.1–7.1.

Results

In the present study, the CLEM method was used to detect Si-FMNP locations in the cell. This protocol presents a method for delivering Si-FMNPs (or specific proteins) into eukaryotic cells (or out of cells) and monitoring their localization and effects on the structures of cellular organelles based on light microscopy and electron microscopy resolutions. Flowcharts of the procedures are presented in Figs. 1 and 2. Confocal microscopy Z-stack images were obtained using a Si-FMNP fluorescence dye, which incorporated Si-FMNPs into the cytosol and nuclei of the HeLa cell (Fig. 4). The cells of interest identified under fluorescence microscopy were well correlated with the low-magnification TEM images (Fig. 5). In addition, the nanoparticles identified in the z-slice fluorescent image were located in the cytosol and nucleus in the high-magnification TEM images (Fig. 6). Based on a 3D

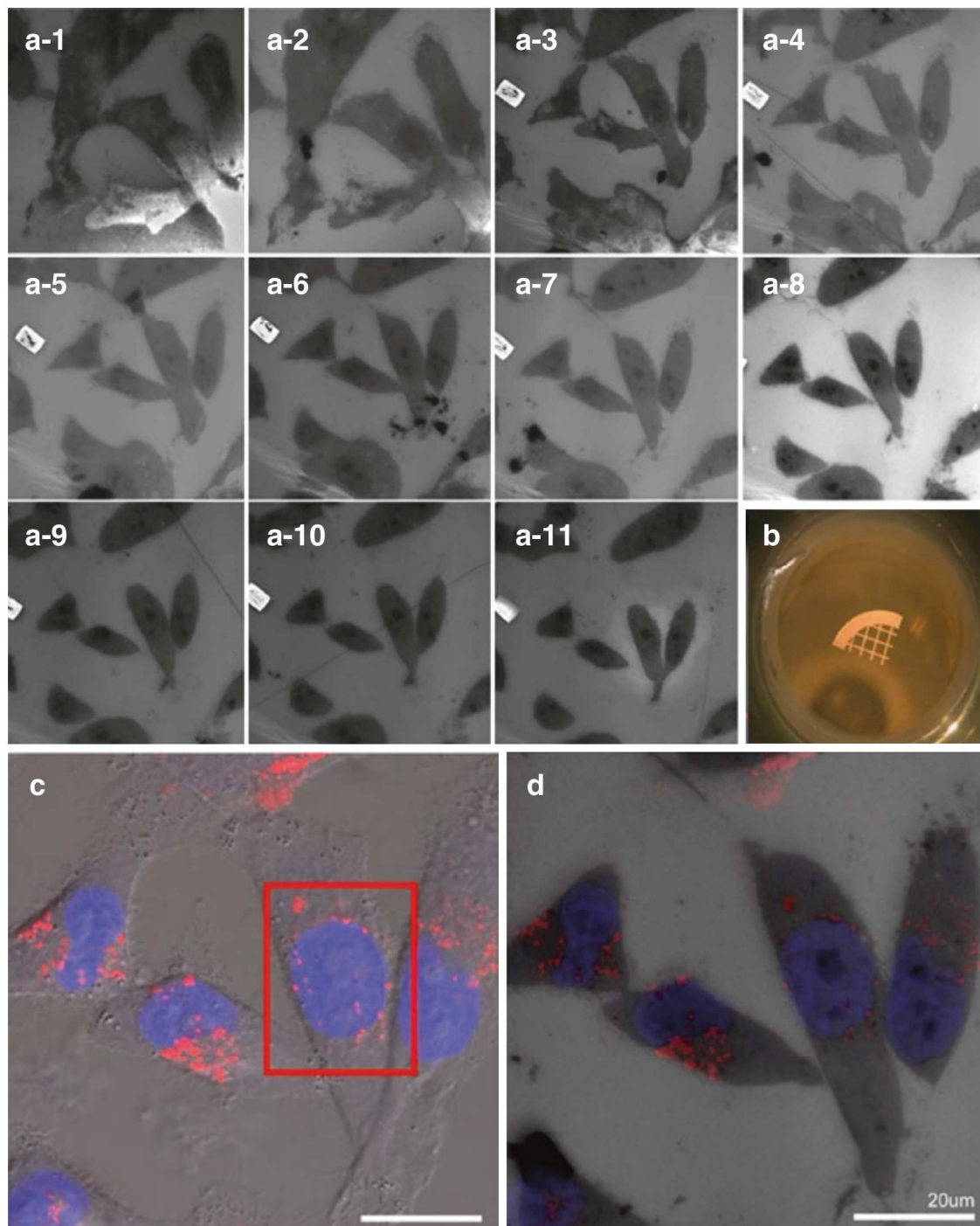


Fig. 5 Low-magnification, low-voltage TEM serial images. **(a1–a11)** Images of serial sections of target cells acquired using low-voltage TEM. Section thickness is 250 nm. **(b)** The gold grid mesh contains target of interest. **(c)** Overlaid image of bright field and the fluorescence signatures of RITC-loaded silica NPs is shown. The red box indicates the cell containing NPs in the nucleus as well as in the cytosol that will be visualized using CLEM in the subsequent step. **(d)** Correlated images of a single section shown in panel A8 and fluorescence. Red and blue fluorescence are originated from Si-FNMP and DAPI staining, respectively

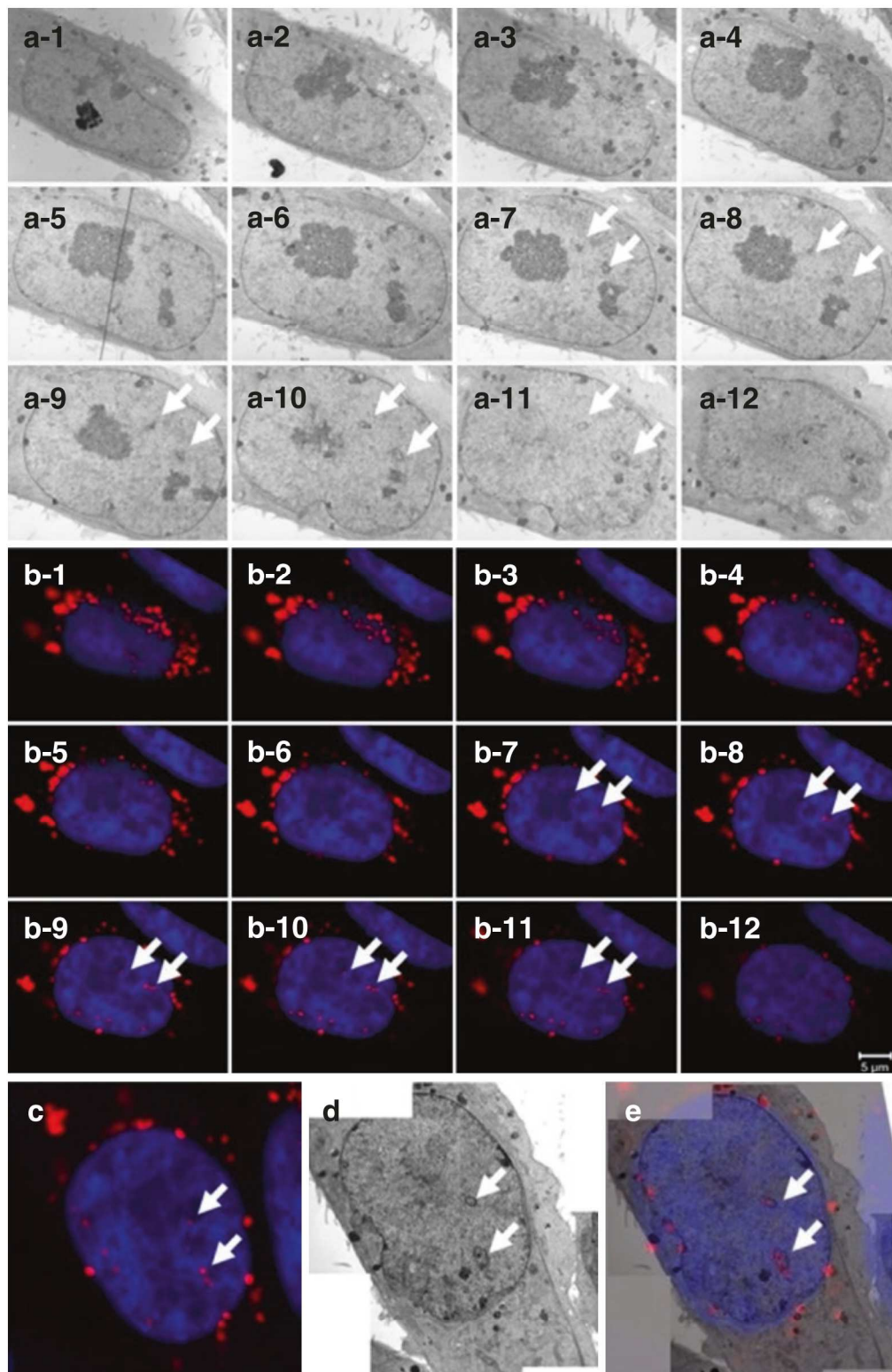


Fig. 6 High-magnification serial images acquired using TEM and z-slice confocal images. (a1–a12) High-magnification serial images acquired using TEM. (b1–b12) Z-slice fluorescent image of the cell nucleus. Optical-slice resolution is 0.5 μm . (c) White arrows indicate Si-FMNPs localized on the nucleus. (d) Electron microscopic image of the target cell corresponding to the fluorescence image shown in C. White arrows indicate an unknown nuclear structure surrounded by membrane. (e) Correlated fluorescence and EM images of C and D are shown. Fluorescence of NPs localization in the nucleus is consistent between the fluorescence microscopy and EM images, although some Si-FMNPs located in the cytosol were not

reconstruction of the serial TEM image, we could identify the exact locations of the Si-FMNP particles in a tunnel-like structure with the nuclear envelope in the nucleus (Fig. 7). To confirm the interaction between the NPs and the cell surface, the position of the cell of interest in the gridded glass-bottom dish was identified (Fig. 8), and the NPs on the cell surface were observed under an SEM microscope (Fig. 9).

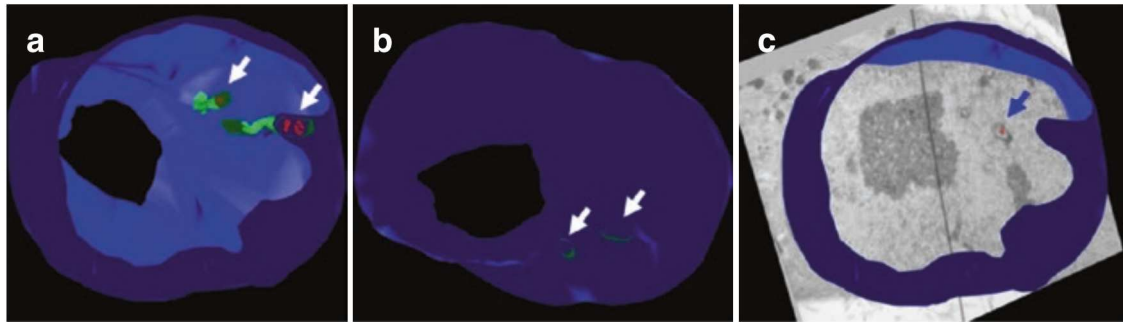


Fig. 7 3D reconstruction image obtained from serial sections of Si-FMNPs incorporated in the nucleus. Surface model rendering of the nuclear membrane (blue), inner tunnel-like structure (green), and nanoparticles (red) was carried out. (a) Views from bottom, (b) from top, and (c) from middle of the sliced images are shown, respectively

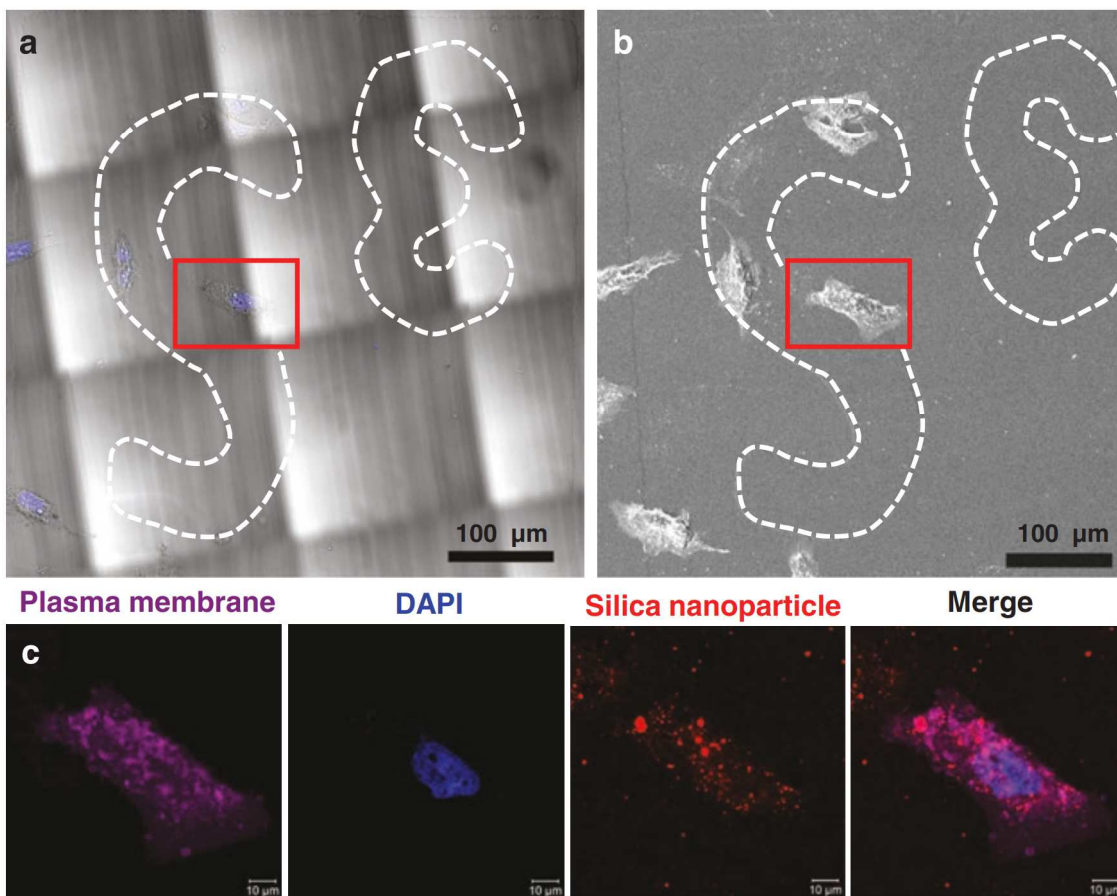


Fig. 8 Identification and selection of cells with silica NPs. (a) A tiled image of Si-FMNP treated HeLa cells on the gridded bottom dish based on confocal microscopy. The red box presents a selected target cell for electron microscopy observation. (b) Low-magnification SEM image. (c) High-resolution fluorescence images of the identical cell shown A and B was obtained through confocal microscopy

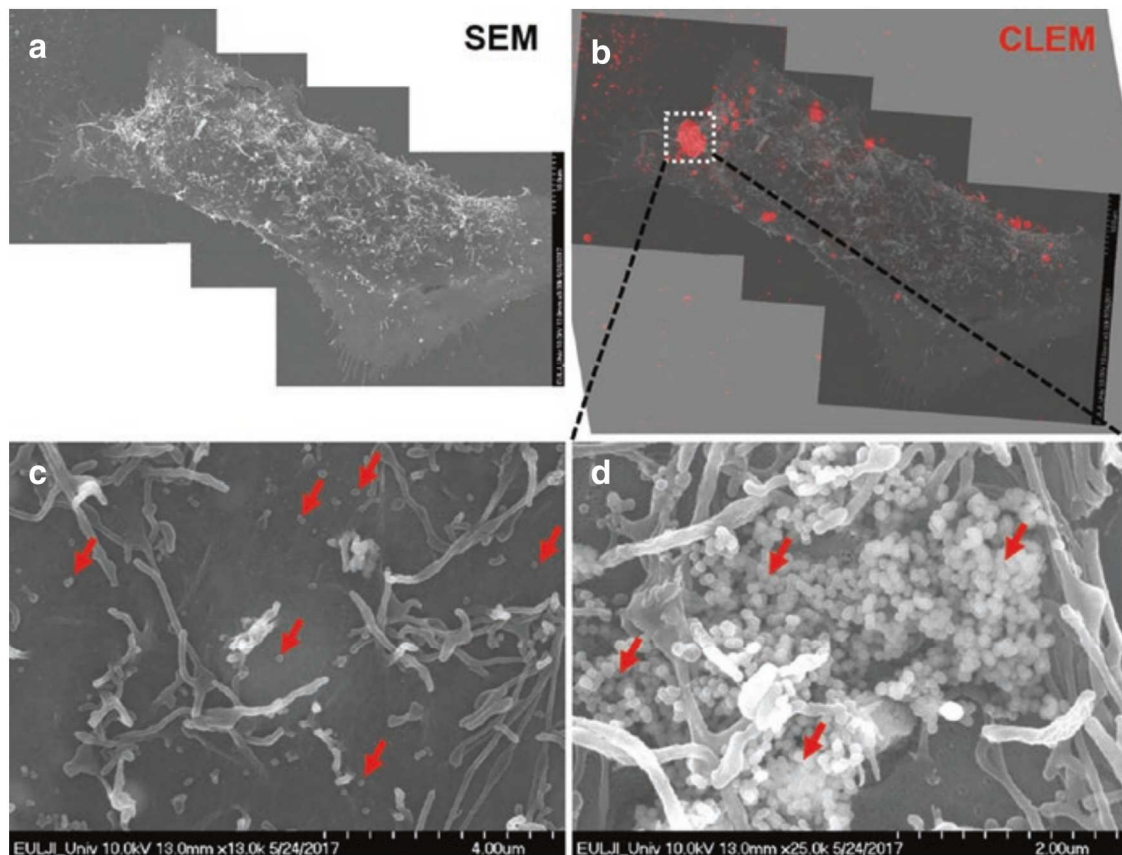


Fig. 9 Correlative micrograph of fluorescence and scanning electron microscopy. **(a)** High-resolution SEM images of the target cell shown in Fig. 8. **(b)** Correlative image from fluorescence microscopy and SEM. **(c)** High-magnification image of a part of the cell surface shown in B, in which single NPs are broadly distributed. **(d)** High-magnification images of the target cell surfaces (dotted line box in **b**). Red arrowheads indicate surface-bound Si-FMNPs

Recently, a genetically modified tag (APEX2) has been developed as a method for identifying the location of a specific protein in a cell at the nanometer scale using EM. We cultured HeLa cells and confirmed the expression of mito-linked APEX2 (mitochondrial matrix protein) in transfected cells (Fig. 10a). A low-magnification EM image showed the APEX2 staining of the mitochondria (Fig. 10c, e) or not (Fig. 10b, d). In high-magnification EM images, the mitochondrial matrix was stained by APEX2, not the intermembrane space (Fig. 10e).

Summary

CLEM is a unique method for the visualization of cellular structures of interest or specific events, which are traced using confocal microscopy observation of a live cell, thus facilitating a detailed EM analysis. Therefore, users can evaluate the specific structures and status of fluorescent probes under EM by providing details of their previous images obtained through confocal microscopy. The procedures and

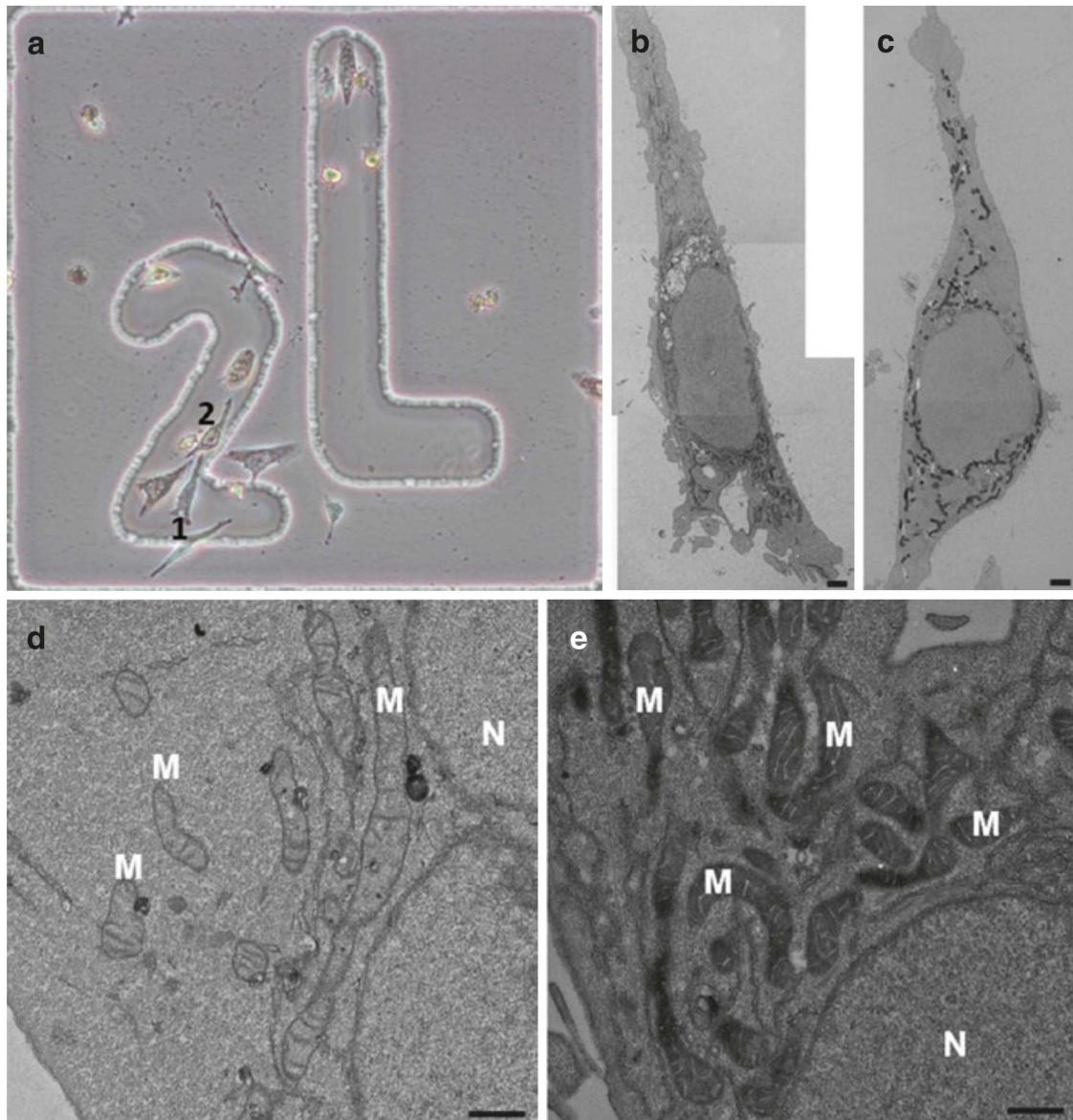


Fig. 10 Genetically encoded reporter gene (APEX2) for correlative light and electron microscopy. **(a)** The phase-contrast images show HeLa cells after DAB staining. The numbers 1 and 2 indicate the two cells to be analyzed. **(b, c)** TEM images of the two cells with low magnification. Dark contrast in the cells indicates that DAB is significantly precipitated mitochondria. Scale bar, 2 μ m. **(d)** High-magnification TEM image of untransfected HeLa cells is shown. The mitochondria with non-DAB precipitated formation is presented. Scale bar, 500 nm. **(e)** TEM image of a transfected HeLa cell is shown. Dark contrast is apparent in the mitochondria matrix and not cristae. Scale bar, 500 nm. M and N stand for the mitochondria and nucleus, respectively

protocol described here provide a detailed example of 3D confocal imaging, fixation, and labeling, in addition to TEM and SEM processing of CLEM specimens. We believe that our method could be applied in the field of convergence science and in the cell biology research community. We also expect it to be a useful platform for further CLEM development and improvement for the evaluation of the efficacy of drug-loaded nanocarriers in various cells and tissues.

Notes

1. Glutaraldehyde and other chemical fixative solutions are highly toxic. Therefore, one must wear protective clothing and work in a fume hood.
2. The mesh grid facilitates the identification of a region of interest. However, the use of a glass dish with a gridded bottom glass dish is convenient.
3. Cell confluence is vital since a full plate makes it difficult to identify the cells of interest under electron microscopy. To find the proper orientation in the EM images, both the shapes of the cells and empty spaces are used as landmarks.
4. Because the cells in the culture dish are dissolved using HMDS, the bottom glass is separated and placed in a glass petri dish (small diameter). The cell surfaces should face upwards.

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References

- Abandowitz HM, Geissinger HD (1975) Preparation of cells from suspensions for correlative scanning electron and interference microscopy. *Histochemistry* 45(2):89–94
- Bykov YS, Cortese M, Briggs JA, Bartenschlager R (2016) Correlative light and electron microscopy methods for the study of virus-cell interactions. *FEBS Lett* 590(13):1877–1895
- Darcy KJ, Staras K, Collinson LM, Goda Y (2006) An ultrastructural readout of fluorescence recovery after photobleaching using correlative light and electron microscopy. *Nat Protoc* 1(2):988–994
- Han S, Raabe M, Hodgson L, Mantell J, Verkade P, Lasser T, Landfester K, Weil T, Lieberwirth I (2019) High-contrast imaging of nanodiamonds in cells by energy filtered and correlative light-electron microscopy: toward a quantitative nanoparticle-cell analysis. *Nano Lett* 19(3):2178–2185
- Horstmann H, Vasileva M, Kuner T (2013) Photooxidation-guided ultrastructural identification and analysis of cells in neuronal tissue labeled with green fluorescent protein. *PLoS One* 8(5):e64764
- Ishikawa-Ankerhold HC, Ankerhold R, Drummen GP (2012) Advanced fluorescence microscopy techniques – FRAP, FLIP, FLAP, FRET and FLIM. *Molecules* 17(4):4047–4132
- Jin R, Zeng C, Zhou M, Chen Y (2016) Atomically precise colloidal metal nanoclusters and nanoparticles: fundamentals and opportunities. *Chem Rev* 116(18):10346–10413
- Kulkarni RV, Setty CM, Sa B (2010) Polyacrylamide-g-alginate-based electrically responsive hydrogel for drug delivery application: synthesis, characterization, and formulation development. *J Appl Polym Sci* 115(2):1180–1188
- Lam SS, Martell JD, Kamer KJ, Deerinck TJ, Ellisman MH, Mootha VK, Ting AY (2015) Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nat Methods* 12(1):51–54
- Martell JD, Deerinck TJ, Sancak Y, Poulos TL, Mootha VK, Sosinsky GE, Ellisman MH, Ting AY (2012) Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. *Nat Biotechnol* 30(11):1143–1148

- Martell JD, Deerinck TJ, Lam SS, Ellisman MH, Ting AY (2017) Electron microscopy using the genetically encoded APEX2 tag in cultured mammalian cells. *Nat Protoc* 12(9):1792–1816
- Pack CG, Song MR, Tae EL, Hiroshima M, Byun KH, Kim JS, Sako Y (2012) Microenvironments and different nanoparticle dynamics in living cells revealed by a standard nanoparticle. *J Control Release* 163(3):315–321
- Pack CG, Yukii H, Toh-e A, Kudo T, Tsuchiya H, Kaiho A, Sakata E, Murata S, Yokosawa H, Sako Y, Baumeister W, Tanaka K, Saeki Y (2014b) Quantitative live-cell imaging reveals spatio-temporal dynamics and cytoplasmic assembly of the 26S proteasome. *Nat Commun* 5:3396
- Pack C-G, Jung M-K, Song M-R, Kim J-S, Han S-S, Sako Y (2014a) Chapter 11 – Use of engineered nanoparticle-based fluorescence methods for live-cell phenomena. In: Cornea A, Conn PM (eds) *Fluorescence microscopy*. Academic, Boston, pp 153–169
- Patra JK, Das G, Fraceto LF, Campos EVR, Rodriguez-Torres MDP, Acosta-Torres LS, Diaz-Torres LA, Grillo R, Swamy MK, Sharma S, Habtemariam S, Shin HS (2018) Nano based drug delivery systems: recent developments and future prospects. *J Nanobiotechnol* 16(1):71
- Radu DR, Lai CY, Jeftinija K, Rowe EW, Jeftinija S, Lin VS (2004) A polyamidoamine dendrimer-capped mesoporous silica nanosphere-based gene transfection reagent. *J Am Chem Soc* 126(41):13216–13217
- Regier MC, Taylor JD, Borczyk T, Yang Y, Pannier AK (2012) Fabrication and characterization of DNA-loaded zein nanospheres. *J Nanobiotechnol* 10:44
- Rieder CL, Bowser SS (1985) Correlative immunofluorescence and electron microscopy on the same section of epon-embedded material. *J Histochem Cytochem* 33(2):165–171
- Sahl SJ, Hell SW, Jakobs S (2017) Fluorescence nanoscopy in cell biology. *Nat Rev Mol Cell Biol* 18(11):685–701
- Sailor MJ, Park JH (2012) Hybrid nanoparticles for detection and treatment of cancer. *Adv Mater* 24(28):3779–3802
- Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, Jin Y, Ellisman MH, Tsien RY (2011) A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. *PLoS Biol* 9(4):e1001041
- van Rijnsoever C, Oorschot V, Klumperman J (2008) Correlative light-electron microscopy (CLEM) combining live-cell imaging and immunolabeling of ultrathin cryosections. *Nat Methods* 5(11):973–980
- Webster RE, Osborn M, Weber K (1978) Visualization of the same PtK2 cytoskeletons by both immunofluorescence and low power electron microscopy. *Exp Cell Res* 117(1):47–61