Biological Specimens Preparation for X-ray Microscopy

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Abstract

In recent decade, x-ray microscopy has become a powerful technique for structure study in biological science by filling the gap between transmission light and electron microscopies in high resolution and high focal depth. However, one of necessary challenge is sample quality evaluation without an x-ray microscope. To deal with this big challenge, transmission electron microscopy has been adapted for the evaluation of sample preparation for x-ray microscopy. The procedures of resin embedding and sectioning for x-ray microscopic sample quality control by transmission electron microscopy have been created in this research.

1 Introduction

X-ray microscopy is a powerful technique for structural biology analysis, which utilizes x-rays to generate the images of structure instead of visible light and electron beam in light and electron microscopes respectively. X-ray microscopy takes advantages above other techniques due to high penetrating depth of x-ray radiation. This particular characteristic makes x-ray microscopy bridge the gap between light and electron microscopies. X-ray microscopy can examine wet, thick, or unstained biological specimens, which are difficult to observe in transmission electron microscopy. Thus, it is a method compatible with living specimen investigation. X-ray microscopy can performs higher resolution due to its shorter wavelength than light microscopy. However, x-ray microscopy also has some disadvantages also. Because of high energy of photon, it can destroy the sample and be harmful from radiation damage. Another drawback is lower resolution comparing to electron microscopy. All comparative information of three microscopic techniques is summarized in Table 1.

Table 1. Comparative information of light, transmission electron, and x-ray microscopies

	LM	**TEM**	**XRM**
Unstained sample	common	difficult	possible
Sample thickness	medium	low	high
Living sample	possible	impossible	possible
Resolution	150 nm	< 0.1 nm	up to 10 nm
Radiation damage	less harmful	serious	violent
Focal depth	medium	low	high

LM; light microscopy, TEM; transmission electron microscopy, XRM; x-ray microscopy

From disadvantages, they become the challenges in x-ray microscopy development research to improve quality of images along with sample compatibility. Since 2013, tender x-rays band have been attractive to increase ability of thick specimen imaging instead of soft x-ray band in ordinary x-ray microscope.[1,2] Tender x-ray microscope can enhance sample accessibility in higher thickness from several microns in soft x-ray microscope to 40-60 microns due to higher penetration. Anyways, it performs poor contrast images because operating photon energy is apart from water window. To improve quality of images, Zernike phase contrast, which is a technique for image enhancement for transparent objects in light microscope, was adapted into tender x-ray microscope, and performed potency in good contrast and resolution and ability for 3D tomographic study. However, only development instrument and measurement research cannot achieve highest quality of images, so sample preparation is also necessary in context to preserve the nature of the structures and enhance quality of sample. In this research, electron microscopy has been adapted for the quality evaluation of x-ray microscopic sample without the need of an x-ray microscope. The cell images were collected from light and electron microscopies in sample quality evaluation purpose.

2 Principles

2.1 Light microscopy

Light microscope is the oldest and original design of microscope. It utilizes visible light illumination and a system of optical lenses to magnify images. However, light microscopy has been developed since the 17th century. There are a lot of modern techniques invented for image enhancement to reveal and characterize the structure of sample. In this research, light microscopy was used for sample checking and method evaluation.

2.1.1 Fluorescence light microscopy

Fluorescence microscopy is an advanced technique for light microscopy staining samples with fluorescent dyes. Some fluorescent dyes can bind specific targets in cellular structures. The fluorescent molecules will absorb unique wavelength excitation photon from light source and emit longer specific wavelength of fluorescent photon. This result can assist scientist determine and clarify the cellular structures from microscopic images. The instrumental diagram of fluorescence microscope is illustrated in Figure 2. In this research, Nile Red and 4',6-diamidino-2-phenylindole (DAPI) were used for staining specimens. Each dye has different specific targets and fluorescent properties. Those are summarized in the following statement.

Nile Red is a lipophilic stain selective to intracellular lipid structure and droplets. It absorbs maximum excitation wavelength at 485 nm and emits maximum fluorescent wavelength at 525 nm in aqueous solution.

DAPI is a fluorescent molecule that can pass through the cell membrane to bind with A and T bases in DNA. It absorbs maximum excitation wavelength at 358 nm, and emits maximum fluorescent wavelength at 461 nm in aqueous solution.

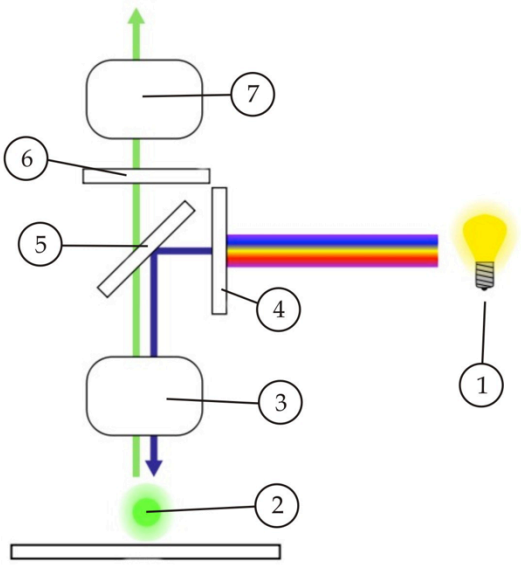


Figure 2. Instrumental diagram of a fluorescence microscope [3]

1. Light source
2. Specimen
3. Objective lens
4. Excitation filter
5. Dichroic mirror
6. Emission filter
7. Eye piece lenses ||

2.1.2 Phase-contrast light microscopy

Phase-contrast imaging is a method to enhance quality images of transparent samples because they have poor light absorption and scattering. As HFF is nearly invisible cell in ordinary light microscope, phase-contrast technique plays an important role in this research. Ordinarily, light transmitting through a sample will be shifted in phase by -90° relative to background light. The basic principle of phase-

contrast is to separate specimen scattered light (yellow vector in Figure 3) from background light (red vector in Figure 3) by making phase change of specimen scattered light, which is typically weak and phase shifted. This reason makes the intensity of foreground light (blue vector in Figure 3) and background nearly equal providing low contrast of image (Figure 3a). When the phase of background light is shifted by -90° shift ring. The phase of specimen and background light become identical (Figure 3b). The phase-changed light will pass through a further gray filter ring to reduce background signal (Figure 3c). In final result, the foreground intensity is much stronger than the background causing the high contrast of image. In addition, phase-contrast method can be applied to electron and x-ray microscopy with the same basic concept of imaging enhancement.

Figure 3. The instrumental diagram of phase contrast microscope[4]

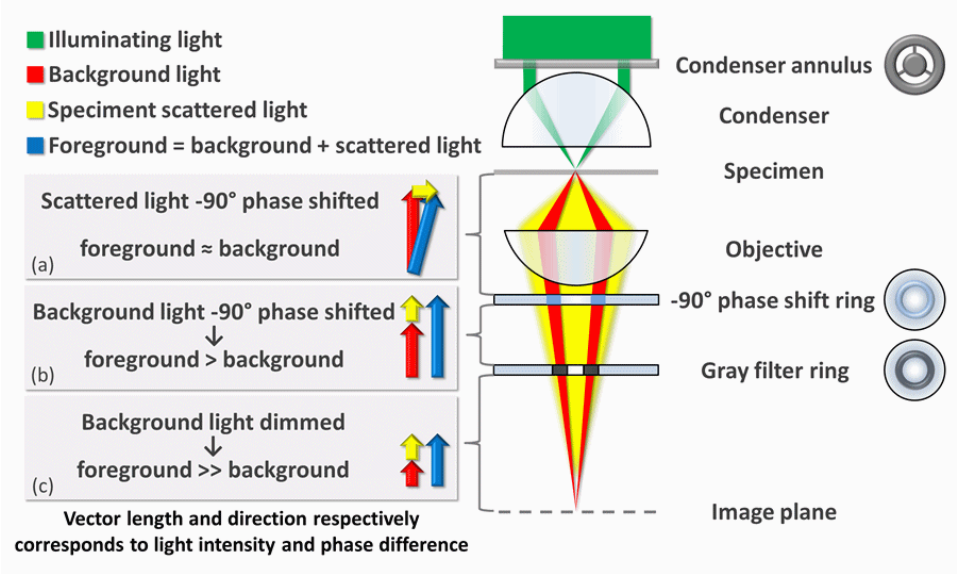
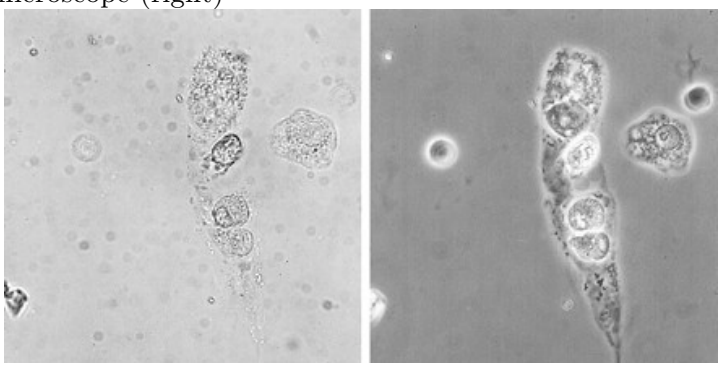


Figure 4. The comparative images between ordinary light microscope (left) and phase contrast light microscope (right)[4]



2.2 Electron microscopy

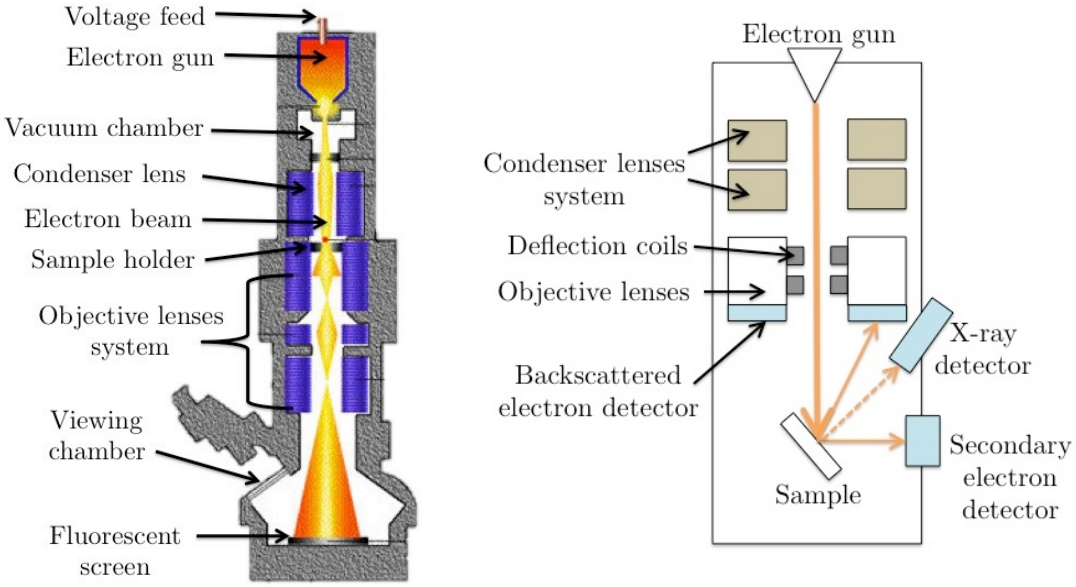
Differ from light microscope, electron microscope magnifies the images by electron beam exposure to the sample instead of visible beam and also replace the system of optical lenses with electromagnetic lenses. Because of the 100,000 times

shorter wavelength than visible light of an electron, it can perform a much greater resolving power than a light microscope.

For instrumental principle, electron microscope still has the same main components as light microscope illustrated in Figure 5. It starts at the filament of electron gun, which is applied with high voltage and emits electron beam thermionically into vacuum chamber inside microscope. Then the electron beam is passing through condenser lens via sample window and objective lenses system respectively before ending at fluorescent screen where the electron beam is transformed into an image of sample. This basic principle becomes transmission electron microscopy (TEM) technique for inside specimen imaging. In advance, electron microscope has been developed to technique for object surface study. The developed method, which illustrated in Figure 5, is to scan electron beam over the sample surface instead of passing through the sample. This technique is called scanning electron microscopy (SEM). Both TEM and SEM are beneficial methods for small object observation in wide range of research.

Although electron microscopy can provide high quality of an image, the complicate sample preparation is also required. Due to low propagating ability of electron beam, it cannot transmit through thick or wet biological specimens. Furthermore electron beam can destroy sample by radiation damage. Hence, living specimens cannot be investigated under the electron microscope, and must be inactivated and dehydrated before examining in electron microscope. These reasons afford the challenge in sample preparation development research to design a method to preserve the structure of specimen as almost same as the living one in order to provide high quality images of exact structure.

Figure 5. The instrumental diagram of transmission electron microscope (TEM) (left), and scanning electron microscope (SEM) (right)



2.3 X-ray microscopy

X-ray microscopy has a basic principle similar to light and electron microscopy, but it uses x-ray instead of visible light and electron beam respectively.

X-ray is an electromagnetic wave corresponding to energy in the range 100 eV to 100 keV. X-rays can be classified by photon energy into three bands. X-rays with energy below 2 keV is called soft x-rays, which have low penetration and could be absorb easily in air. The x-rays with energy between 2-5 keV are classified as tender x-rays while the x-rays with energy above 5 keV are classified as hard x-rays. Each x-ray band can be utilized in various applications. Due to similar wavelength to size of atom, hard x-rays are employed for structure determination of crystal in crystallography research. By contrast, soft x-rays are mostly applied in x-ray microscopy because soft x-rays can achieve good contrast images of unstained, wet, and several micrometers thick specimens in water window. The water window, which is a range of soft x-ray energy between K-absorption edge of oxygen and carbon at 284 and 540 eV respectively, is presented in Figure 7. For soft x-rays with energy among the edges, water is transparent to these x-rays while carbon, nitrogen, and other elements found in the structure of biological specimens are still absorbing. For this reason, soft x-rays in water window range can be utilized for living specimen study without chemical fixation or dehydration that is required in electron microscopy. Tender x-rays cannot achieve high contrast images like soft x-rays in the water window though. This is a big challenge to make advantageous high contrast images of thicker specimens along with equivalent or better resolution. Thus, Zernike phase-contrast principle in light microscopy has been applied for tender x-rays resulting a better contrast image, which could be developed to x-ray microtomography for 3D structure by 360° rotated sample imaging. All of these applications can create new challenge and novel discovery in biological science.

For instrumental principle shown in Figure 6, it starts at x-rays source, e.g. x-ray tube or synchrotron light source, which generates x-rays propagating to detector *via* condenser zone plate, sample window chamber, and objective zone plate before ending at an x-ray camera. The transmitting x-ray is converted into electrical signal by detector. The electrical signal is analyzed and reconstructed into an image.

Figure 6. The instrumental set up scheme of x-ray Zernike phase-contrast microscope at beamline P11, PETRA III, DESY[5]

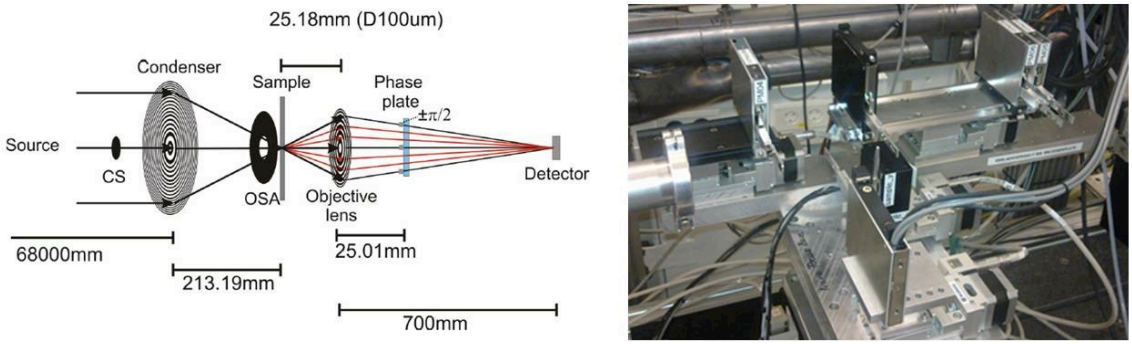
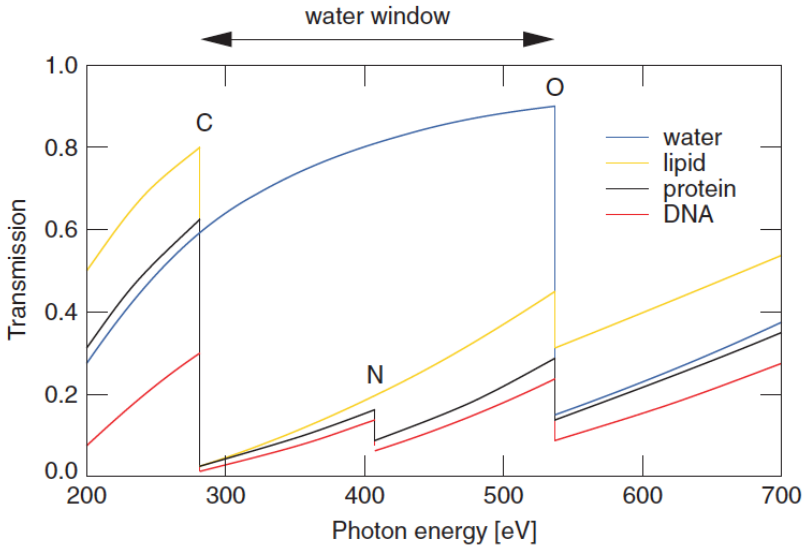


Figure 7. Transmission curves of 1 mm-thick biological materials in the water window between approximately 284 and 540 eV of photon energy[6]



3.1 Cell preparation

3.1.1 Cell initiation

Human Foreskin Fibroblasts (HFF) was used as the sample for imaging. The cells were cultured by adherent cell culture. The cell line was initiated from the cryopreserved cells in 10% DMSO by warming to 37°C for starting culture. Afterward, the cells were diluted with Dulbecco's Modified Eagles Medium (DMEM) containing foetal bovine serum (FBS), glutamax solution, and antibiotics (Penicilin & Streptomycin) in T75 tissue culture flask. The cell line was incubated overnight under standard conditions (37°C, 5% CO_2). After initiation, the cells were diluted with fresh medium in stock culture. Then, the medium was exchanged in next day.

3.1.2 Sub-culturing and monolayer cell deposition

The cells were removed the medium and washed with PBS. The adherent cells were detached by trypsin-EDTA solution. The flask was rocked to ensure that the solution covered the entire surface and incubated under at 37°C for 5 minutes. The fresh medium was added into the flask and mixed with the cells. These freed cells might be sub-cultured by divided into the new flasks or grown as monolayer tissue onto a flat surface window for microscopic analysis. The window might be a glass coverslip or a silicon nitride (SiN) membrane. The cell seeding was accomplished by transferring the window into 6-well or 96-well plate, which filled with the mixture of cells and medium, for glass coverslip and SiN membrane respectively. The plates were incubated under standard conditions for at least 4 hours.

3.1.3 Cell fixation

When the cells grew and increased to sufficient quantity seen under light microscope, the cells were inactivated at room temperature by chemical fixative, paraformaldehyde (PFA) and gluteraldehyde (GA). In each fixative, the cells need be rinsed with PBS before changing fixative. Due to auto-fluorescence of GA, the sample was fixed with GA only for electron microscopy purpose. After finishing fixation, the coverslips were washed again with PBS twice to clean fixatives.

3.2 Light fluorescence microscopic imaging

In this research, Nile Red and DAPI were used as fluorescent dyes for HFF cells staining. The stock solution of Nile Red and DAPI need be prepared. Nile Red was dissolved in DMSO (1 mM/mL) and must be kept in dark place and tight seal. DAPI was dissolved in distilled water (1 mg/mL) kept in dark place at -25 ºC. The cells were incubated in dyes solution for 15 minutes. Nile Red was 100 times diluted in PBS, and DAPI was 1,000 times diluted in PBS. After incubating in dye solution, the cells were rinsed with twice PBS and distilled water. In next step, Mowiol, the mixture of polyvinyl alcohol and glycerin, was used as for mounting medium. It was dropped on *iso*-propanol cleaned glass slide. Then the cells on coverslip were placed toward a Mowiol drop. Afterward, the sample was dried at heating plate overnight. The dyed cells were photographed in Nikon Ti-E fluorescence microscope.

3.3 Electron microscopic imaging

3.3.1 Heavy metal staining

To provide a good contrast of structures in an image, heavy metals stained into cell components to improve scattering and absorptivity of a sample leading to an enhanced image. In this research, 1% osmium tetraoxide (OsO_4) in 0.1 M sodium phosphate buffer pH 7.3 was used as staining chemical at room temperature for 15 minutes in the humid controlled chamber. Again, the stained cells need be rinsed with PBS at least twice and followed with deionized water once.

3.3.2 Dehydration and infiltration

The cells were dehydrated in 50%/70%/90% and twice absolute ethanol series for 10 minutes in each concentration. Eventually, entire cellular water was substituted with ethanol. The dehydrated samples were removed liquid by supercritical drying. The cells were immersed in 50%/70%/90% and twice absolute freon 113/ethanol series for 10 minutes each, and then dried in incubator at 30ºC overnight. The dried cells for TEM might be incubated with three changes of LR white (Medium grade, London Resin Company, London, UK) for 1 hour/change. In this research, 1:2 mixture of ethanol:LR White infiltrated and non-infiltrated samples were also examined in order to study the influence of pre-infiltration process toward the imaging quality.

3.3.3 Sample embedding and sectioning

The monolayer cell on window would be embedded with resin to be solidified for fine sectioning. LR White, an aromatic acrylic resin mixture, was used as embedding medium. The procedure of resin embedding is illustrated in Figure 9. (a) LR white accelerator was prepared as a thin layer on the glass slides by blood smear technique. Dry gelatin capsules were laid onto the accelerator to immerse the rim of a capsule. (b) The capsules were turn around and three-quarters filled with LR white avoiding toughing the accelerator on the rim. (c) The coverslips or SiN membranes windows were immersed in liquid resin as infiltrated monolayer and placed upside-

Figure 8. Photo and cross-section diagram of silicon-nitride membrane window equipped on silicon aperture frame

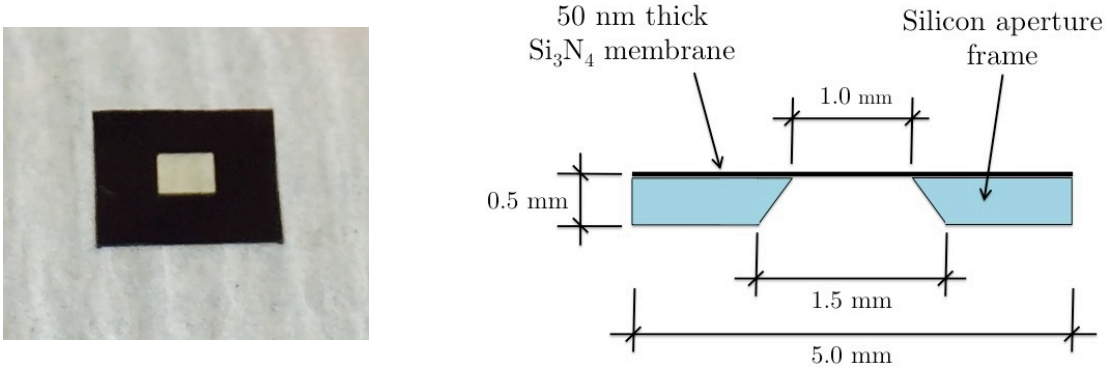
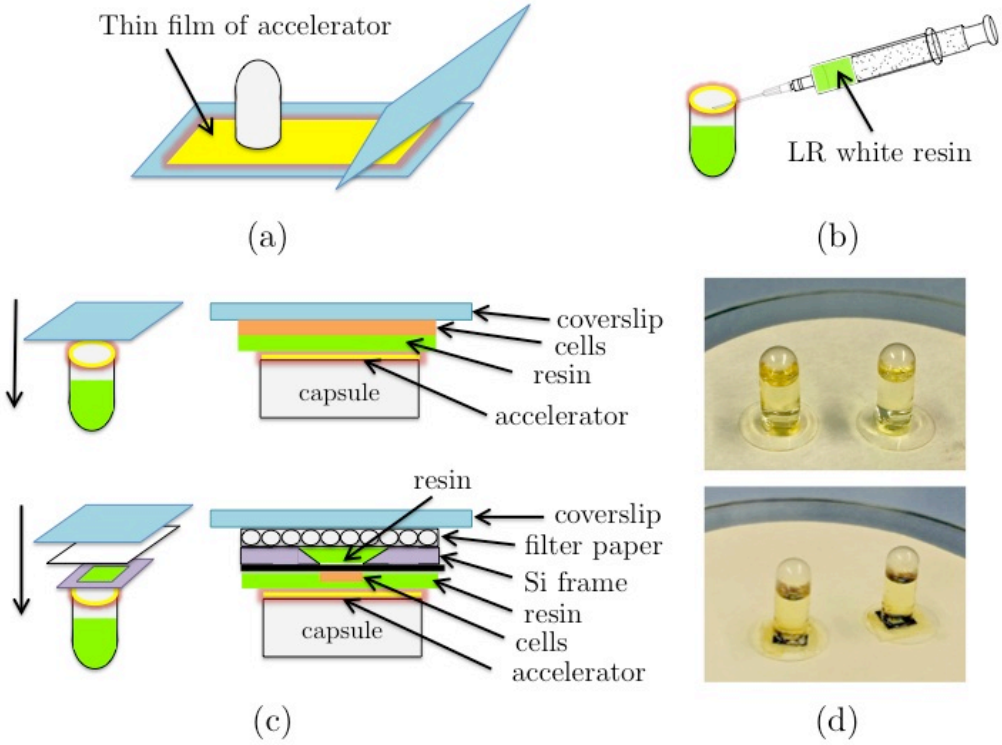


Figure 9. The demonstration of flat embedding cells grown on coverslip or SiN membrane in LR white resin

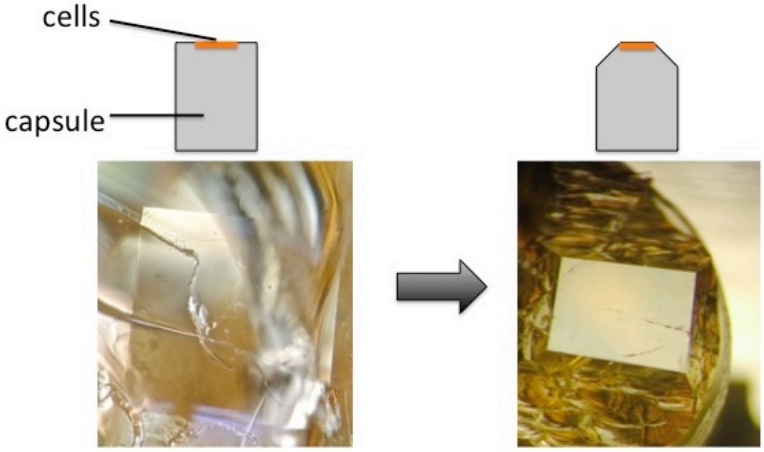


down onto the capsule rim. The accelerator induces the resin monolayer on cell surface to polymerize causing the window tightly fixed to the capsule after leaving for 30 minutes. In case of SiN membrane, LR white was added into the hole of silicon aperture frame to support to membrane. A piece of resin dipped filter paper was placed to close the chamber window. Then, a glass coverslip was put on the top of paper. (d) The resin fluid filled capsules attached tightly with windows were turn around again and placed on the filter paper and polymerized by heat at 50°C within 24 hours. When the resin was polymerized and became whole solid, the capsules were popped off in liquid nitrogen.

When the capsule was separated from SiN membrane, the cell deposited surface was trimmed to a pyramidal shape by ultra-microtome knife. The trimming

method is shown in Figure 10. A trimmed capsule was sectioned into ultrathin sections (60-70 nm thick) by a diamond knife.

Figure 10. Trimming method of capsule before sectioning with diamond knife



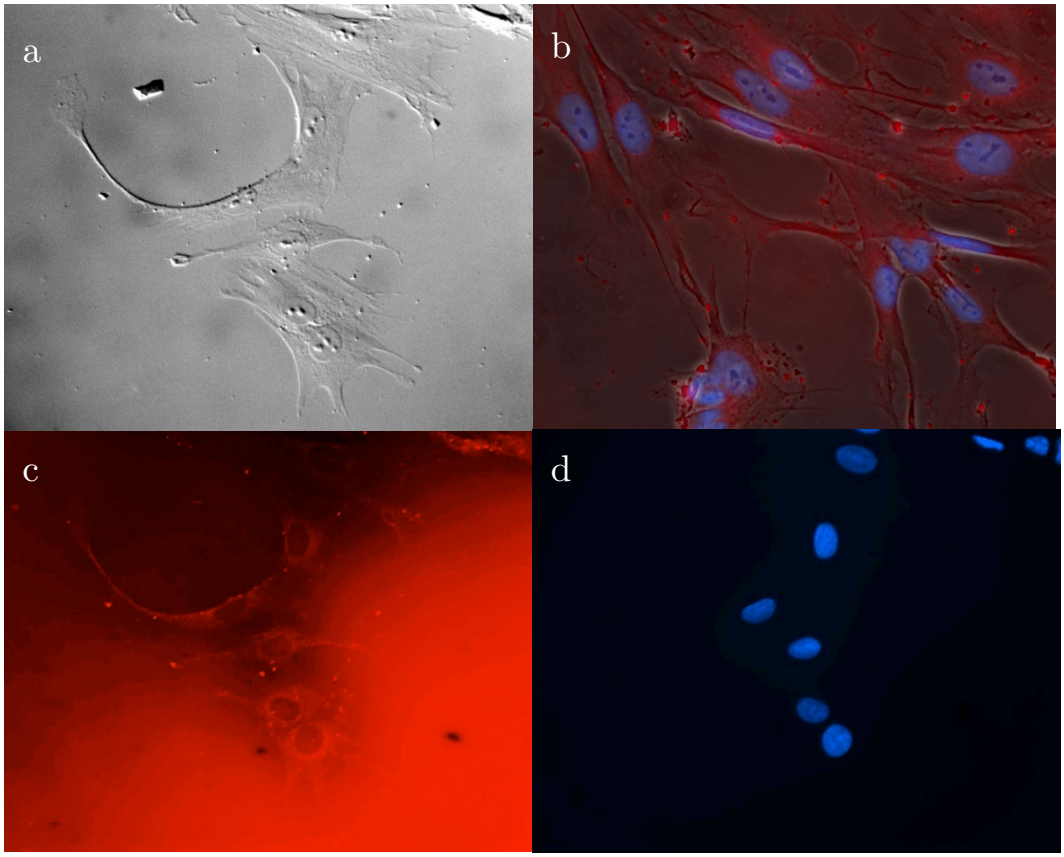
3.3.4 Measurement

Before examining in electron microscope, post-staining with uranyl acetate is necessary to be done in order to increase contrast of an image. The samples were photographed in scanning electron microscopes at Heinrich-Pette-Institut, Hamburg, Germany.

4 Results and discussion

4.1 Cell images in light microscope

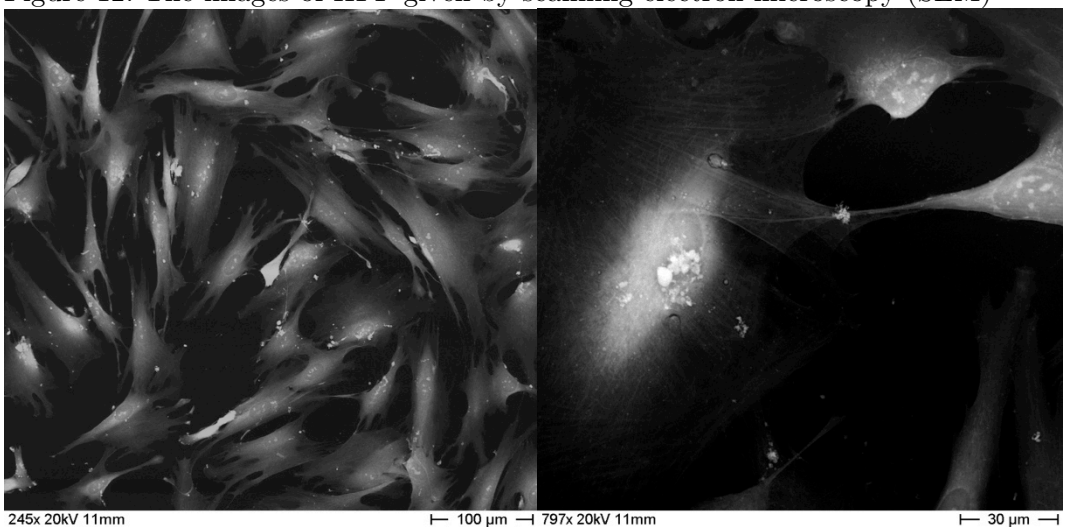
Figure 11. (a) Phase-contrast image (b) Combined channel image (c) Nile Red channel image (d) DAPI channel image



After the cells were fixed and stained with fluorescence dyes, Nile Red and DAPI, the stained sample was inspected in fluorescence light microscope. The photographs of samples are presented in Figure in different channels of fluorescence light microscopy. Thus cell organelles were determined by specific staining dyes. For Nile red dye, it is specific to a carbonyl group of intracellular lipid. All lipid-rich structures, e.g. cell membrane, were labeled as red emission at 485 nm excitation wavelength. Meanwhile, DAPI is specific to A-T bases in DNA structure, so DNA in nucleus was labeled as bright blue fluorescence at 358 nm excitation wavelength. Both of fluorescence channels can be combined together by mean of structure labeling. When comparing between phase contrast light microscopic image Figure 11a and combined channel fluorescence light microscopic image Figure 11b, it is obvious to be seen that fluorescence light microscopy is a useful technique to distinguish the structures in a cell by a labeling image.

4.2 Cell images in scanning electron microscope

Figure 12. The images of HFF given by scanning electron microscopy (SEM)

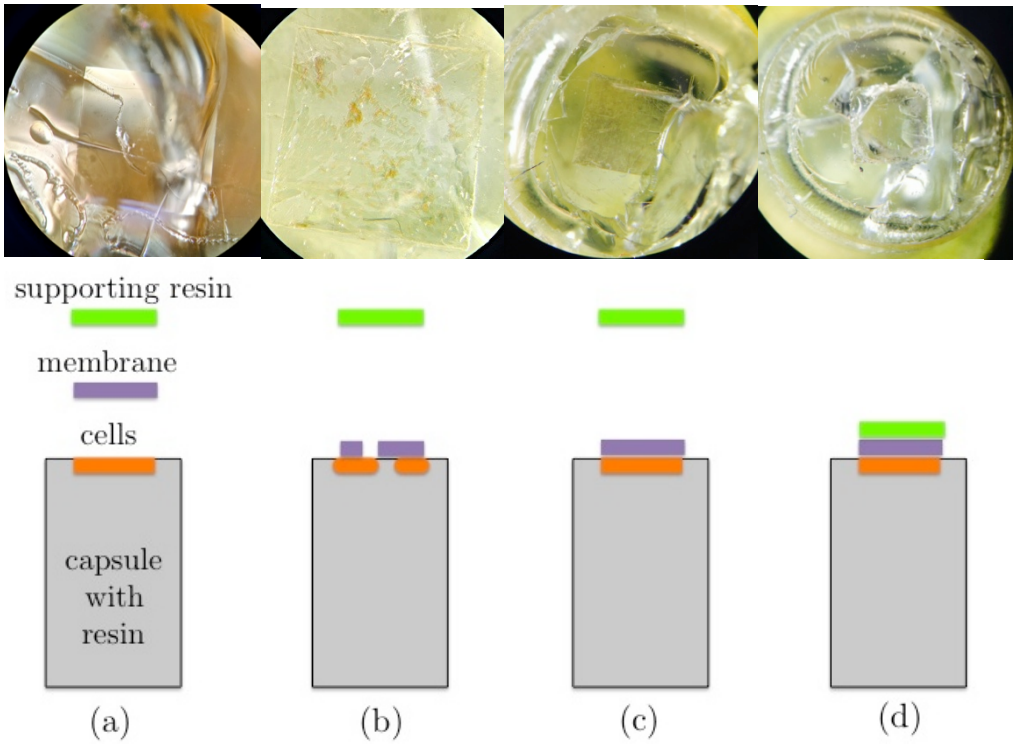


Sample preparation was examined in SEM to evaluate its quality for x-ray microscopy. From the results in Figure 12, HFF sample was ready for x-ray microscopy because the cell structure looks firm and the organelles can be still seen clearly. However, SEM images have lower resolution in comparison to TEM. SEM can be used for elementary evaluation of sample preparation for x-ray microscopy. For further evaluation, TEM is required for final determination.

4.3 New method for resin embedding

By following procedure in section 3, capsular resin embedding was successful to adapt for SiN membrane. The resulting resin surface has been shown in Figure 13a, which shown the cells deposited in resin and completely separated from SiN membrane. Anyways, there were several problems during adaptation for SiN membrane illustrated in Figure 13b-d. (a) This is a good sample that the cells were deposited in resin without SiN membrane and supporting resin attached on cell surface. (b) This sample has severe damaged SiN membrane. The damage occurred

Figure 13. Photos of resin surface of capsule in different cases



from too rapid dipping the capsule into liquid nitrogen. (c) The SiN membrane was not separated from the capsule. It still covered the cell surface on resin when making sample embedding without filter paper support. (d) The supporting resin cannot be popped out and still attached cells and membrane onto resin when the mixture of accelerator and resin (2 μL/mL) was used as supporting resin. Therefore, sample preparation for electron microscopic evaluation of x-ray microscopy sample need be operated carefully.

5 Conclusion and outlooks

The resin capsular embedding method has been successfully adapted for Si_3N_4 membranes although SiN is very sensitive to toughing and impact. Anyway, the procedure of resin embedding of SiN membrane need be highly attended in every step. When resin embedding and sectioning was successful, this prepared sample can be examined in TEM, which evaluates sample quality for x-ray microscopy. For this EM sample quality control, it can save the time for an x-ray experiment because the samples have been checked before carried out in x-ray microscope. Furthermore, the developed method could be used alternatively for the evaluation of new sample preparation. Thus, the sample of x-ray microscopy can be evaluated along with EM by developed procedure.

For future research, cryogenic fixation has interesting potency to inactivate sample cells avoiding toxic chemicals consumption by fixing cells at liquid nitrogen temperature. [7,8] This method might be followed with freeze-drying dehydration that cellular water sublimation in cryogenic and vacuum conditions. In spite of several days spent for drying cell, it is environmental friendly technique in decline of organic solvent use with equal image quality.

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