GENERAL INFORMATION

The Microscopy Society of America (MSA), the world’s largest professional association of microscopists, provides the only certification of technologists in biological transmission electron microscopy available in the Americas. The program was initiated in 1978 to establish standards of technical skills. In addition to insuring employers that certified technologists are technically proficient, certification can be important in determining job classification, salary level, and potential for advancement or promotion. Many consider certification to be a key benchmark in their professional development.

The program is administered by the Certification Board which is appointed by the Council of the Society. The Board develops regulations, formulates and evaluates examinations, and interprets policies.

Individuals with the requisite educational and/or occupational qualifications can attain certification by completing and passing both written and practical examinations. Two examination cycles are offered each year. Complete regulations and an application form appear on the following pages.

The initial period of certification is one year, the calendar year indicated on the certificate furnished to all successful candidates. Certification may be renewed on a 10-year cycle by payment of the appropriate fee ($75 for MSA members, $150 for non-members). Certified Technologists who allow their certification to lapse for one year may have it reinstated by paying the appropriate fees; if certification lapses for two or more years, the technologist must submit a new application and take both written and practical examinations again.

Communication with the Society, about certification, should be addressed to:

Microscopy Society of America
Certification Board
11130 Sunrise Valley Rd., Suite 350
Reston, VA 20191
Phone: (703) 234-4115

1Before January 1, 1993, the Microscopy Society of America was the Electron Microscopy
Society of America (EMSA). “Electron” was dropped from the name to reflect the Society’s broadened scope that has come to include all kinds of microscopy and microanalysis. Nevertheless, most of the membership and scientific program still is concerned primarily with electron microscopy.

2 The Society reserves the right to modify these and other fees from time to time to reflect changes in service, dues, etc.

APPLICATION AND REQUIREMENTS

An application for certification consists of:

1. A completed application form (included in this package).
2. An application fee of $75.00 for MSA members or $150.00 for non-members.
3. Transcripts and/or documentation of ONE of the following:

Two years (60 credits) college or equivalent education, including at least 4 semesters of science that include chemistry, physics, biology, and mathematics and two semesters transmission electron microscopy (TEM). The TEM course must include extensive hands-on experience in sample preparation and microscope operation

OR

One year (30 credits) college or equivalent education, including at least one semester of laboratory courses each in chemistry and physics AND one year of recent full-time work experience doing biological TEM, as a volunteer, internship or paid employee.

OR

A high school diploma AND two years of recent full-time work experience doing biological TEM

OR

Three years of recent full-time work experience doing biological TEM

4. Letters of recommendation from two (2) experienced electron microscopists in supervisory positions having substantial records of research publication. At least one of them must be a member of MSA. The letters may either be enclosed with the application or sent separately, but the application will not be considered complete until the letters are received by the Society.

All application materials must be sent to the Association Management Office:
Microscopy Society of America
Certification Board
11130 Sunrise Valley Rd., Suite 350
Reston, VA 20191

Applicants are responsible for seeing that all requirements are sent in time and should check with the Office to make sure their applications are complete. Applications completed after the deadline date will automatically be considered for the next examination cycle.

Completed applications are evaluated by the Certification Board Chair to determine whether the applicant is qualified to take the examinations. Applicants not approved for examination will receive a written explanation and their certification application fee will be refunded (although MSA dues, if any, will not be refunded). Fees for applicants approved for
examination are not refundable.

3Application for membership in MSA is separate from application for certification. Candidates for certification may pay the lower member’s fee by submitting an application for membership, along with one year's annual MSA dues, at the same time they submit the application and fee for certification.

4Payment can be by check (US funds, drawn on a US bank) payable to MSA, or by credit card (Visa or Master Card only). If paying by credit card, supply the complete credit card number and expiration date.

5“recent” is interpreted to mean within the five years prior to application.

EXAMINATIONS: GENERAL
Candidates whose applications are approved must pass both a written and a practical examination in order to be certified. The candidate must pass the written exam before s/he may submit the materials for the practical exam. Both examinations usually are taken during the same cycle in which application was made; however, candidates may request deferring either or both examinations until the next cycle. Candidates who fail an examination in the cycle in which they applied may take it again in the next cycle without penalty.

All requirements for certification must be completed by the cycle after the one in which application is made. Otherwise the candidate must submit a new application, including the application fee and letters of recommendation (transcripts need not be re-submitted unless they have become outdated). Examinations taken prior to re-application must be taken again, even if previously passed.

Written Examination
The written examination is of the objective type (multiple choice, true-false, etc.); three hours are allotted for completion of the written examination. In most cases the examination is conducted in or near the candidate's home institution. A score of 80% is required to pass. The material covered includes:

   A. Instrumentation including electron optics (approx. 25%)
   B. Tissue processing (fixations, resin chemistry etc.) (approx. 25%)
   C. Sectioning and staining (approx. 15%)
   D. Special techniques  (Immuno, shadowing, cryo, etc.) and imaging (approx. 20%)
   E. General: chemistry, safety (approx. 15%)
Written Examination Study Syllabus

A. Instrumentation
Accessory Equipment: Principles, components, alignment and routine maintenance of:
- Ultramicrotomes,
- Knifemakers
- Light microscopes
- Transmission electron microscope fundamentals:
  - Operation; illumination; imaging systems; focusing; maintenance; test specimens; astigmatism; resolution, calibration, contamination
- Scanning electron microscopes: general principles; operation
- Vacuum systems:
  - Vacuum evaporator, sputter coaters, mechanical, diffusion, turbomolecular, ion pumps, vacuum gauges

Other Lab Equipment:
- Incubators
- Ovens
- Balances
- pH meters
- Osmometers
- Centrifuges
- Photographic techniques (digital and film)

B. Sample/Tissue procurement for TEM processing
Fixation & Processing
- General principles and purpose
- Types, composition, & preparation [glutaraldehyde, paraformaldehyde, OsO4, KMnO4 and others]
- Buffers [eg: phosphate, cacodylate, PIPES, HEPES, s-collidine, veronal- acetate];
- Factors affecting fixation [fixative concentration, time, temperature, pH, osmolarity, buffer, additives, penetration]
- Methods of fixation [immersion, perfusion, vapor]
- Criteria for good fixation
- Washing: general principles and purpose
- En bloc staining
- Dehydration: general principles and purpose
- Dehydrating agents [ethanol, acetone, ethylene glycol, propylene oxide, acetonitrile]
- Factors affecting dehydration [concentration, time, temperature]
- Infiltration: general principles and purpose
- Embedding: general principles and purpose
- Types, composition and preparation of plastics [acrylics, polyesters, epoxies, catalysts, hardeners, plasticizers]
- Methods of embedding [capsules, flat, cell culture, vacuum]
- Polymerization: general principles and purpose
- Safety
C. Sectioning and Staining
Sectioning: general principles and purpose
- Block Preparation: trimming; facing; re-mounting
- Knife preparation: glass breaking, inspection, troughs (boats)
- Diamond knives: use & handling
- Grid Preparation: types; cleaning; coating [Formvar, Butvar, collodion, carbon]
- “Thick” (semi-thin) sectioning; collection, mounting
- Thin sectioning: orientation, flotation [liquid and meniscus], flattening, collection, thickness [interference colors], problems, factors affecting quality

Staining: General principles and purpose
- Thick Section Staining: Toluidine blue-O, methylene blue, Paragon, azure II, Giemsa
- Thin sections: specific stains [uranyl acetate, lead citrate, phosphotungstic acid, osmium, ruthenium, silver] factors affecting staining quality
- Safety

D. Digital Imaging/ Power Point
- General principles and purpose.
- Image processing,
- Use of computers
- Illustrations: labeling, magnifications

E. Special Techniques
- Negative staining
- Shadow casting and replication
- Cytochemistry and immunolocalization
- High Pressure freezing and freeze substitution

F. General
- Basic cytology, cell morphology, ultrastructure
- Reagents: solvents, solutions, normality, molarity, percentage, acids, bases, salts
- Cleanliness: glassware, distilled and deionized water
- Basic math: metric system, trigonometry, measurements
- Safety: radiation, chemical, biological, fire
Written Examination
Sample Questions (from past examinations)

Multiple Choice

1. If a cell structure is 60 mm long on a micrograph at 20,000X, its actual length is:
   (a) 6 um
   (b) 3 um
   (c) 2 um
   (d) 0.33 um
   (e) 0.16 um

2. Proper lab attire includes:
   (a) lab coat or jacket
   (b) open toed shoes or sandals
   (c) full length slacks
   (d) shorts
   (e) a and c above

3. Negative staining is often done with:
   (a) lead citrate
   (b) uranyl acetate
   (c) phosphotungstic acid
   (d) Toluidine blue-O
   (e) b and c above

4. Astigmatism in a TEM can be caused by:
   (a) contamination of an aperture
   (b) improperly aligned filament
   (c) a vacuum leak in the camera chamber
   (d) a bent grid
   (e) b and c above

5. How much 25% glutaraldehyde is needed to make 50 ml of 3% glutaraldehyde?
   (a) 5 ml
   (b) 10 ml
   (c) 6 ml
   (d) 3 ml
   (e) 2.5 ml

Practical Examination
The practical examination consists of preparing blocks, sections, and micrographs from three different samples/tissues and submitting them for evaluation by two (or sometimes three) members of the Certification Board having experience with specimens similar to those submitted. The examiners base their scoring on the usability of the specimens and grids in everyday practice in a research or clinical setting. Thus, the work submitted should represent the candidate’s BEST work. Material submitted should be publication quality and should include image labels that identify key features of the tissues used. Procedures should be written so that anyone familiar with biological electron microscopy procedures could replicate the work. The relative weight given to various
aspects of the submitted material is indicated on the MSA Practical Exam Grading Sheet included in this packet. An average (mean) score of 80 is required to pass.

All work must be done by the candidate alone. However, a supervisor or other qualified individual may assist in obtaining the gross tissue specimens. A signed Pledge of Independent Workmanship (included in this package) must accompany the examination materials. Normal (not pathological and not human) materials and common processing methods should be used.

**Identify all submitted materials with the examination ID number** you were assigned when your application was approved. *Do not label items with either your name or your lab or institution’s name.*

The bullet-points outlined in the syllabus above are indicative of the grading points used in evaluating the practical exam materials. ALL aspects of sample preparation and presentation are considered.

Detailed instructions follow:

1. Prepare three different tissues for transmission electron microscopy, from fixation through sectioning and uranyl acetate-lead staining, on uncoated 200 or 300 mesh copper grids. The tissues may be plant, animal, cell culture or microorganism, as the candidate chooses, except that at least one tissue must be from a mammal, cell culture or higher plant. *Be aware that the embedding resin you choose will affect the quality of your final images.*

2. Submit the following:
   a. one trimmed tissue block from each tissue
   b. four grids with good quality sections cut from each of the submitted blocks
   c. one slide of “thick” (ca. 1 um) sections for light microscopy, appropriately stained, from each submitted block
   d. a detailed description, no longer than one page for each tissue, of the preparation methods used. **Procedures should be written so anyone else could replicate the work.** Be sure to indicate whether a glass or diamond knife was used. This should be modeled after the “Materials and Methods” sections of refereed journals.
   e. There are three acceptable methods to submit images based on the technology the applicant has available to them in their lab. Submit six (6) images of each sample at 3 magnifications within the microscope magnification range of 2,500x to 30,000. Submit at least one at low magnification (survey), at least one at intermediate magnification featuring a single cell, and at least one at higher magnification showing subcellular/organelle features). Image data may be submitted on a CD, DVD or flash drive.

   1. **Digital Image Submission**
      A. Submit raw images as acquired at the microscope in a folder labeled “raw images”. Create a subfolder for each sample. If images are acquired with proprietary software, submit raw images as acquired and also submit raw images in TIFF format in a file labeled “raw images TIFF”.
      B. In a file labeled “annotated images” create a subfolder for each sample. Annotated images must be submitted as TIFF files. Annotated images must include a scale bar.
      C. Create a power point presentation with two images from each sample using the annotated images. Include a figure legend. Do not use any “special effects”.
2. **Photographic Film/Print Submission** (for labs without digital acquisition or scanner only)
   A. Submit original negatives
   B. Submit an annotated 8x10 photographic print of each micrograph. Include a scale bar on each print and a figure legend.

3. **Hybrid Film and Digital Submission**
   A. Record images on film and submit all negatives.
   B. Scan images into digital format as TIFF images. Place in a folder labeled “scanned raw images”.
   C. Follow steps B & C Digital Image Submission above.

   **In the Methods Section:**
   - Identify the digital camera; manufacturer, model and pixel array (eg: 2K x 2K; 11 megapixel).
   - For film/print images, show how you calculated the length of the scale bar for each magnification used. Use three different magnifications.
   - Identify the scanner; manufacturer, model pixel array and dpi, if used.
   - Create a power point presentation with two images from each sample using the annotated images. Include a figure legend. Do not use any “special effects”.

   f. Complete figure legends for each micrograph should be printed on a separate sheet. They should be concise (journal style: e.g. *Microscopy and Microanalysis*) and should describe any labeled structures and scale bars that appear on the micrographs.

3. Separate the grids into three groups of four and place them in a grid box (slide-type preferred) secured with a rubber band or tape. We recommend packing all materials in a sturdy box or padded shipping envelope. Use packing material so that the contents can’t shift during transport.

4. Send all materials (do not forget the *Pledge of Independent Workmanship*) to the **Chair of the Certification Board to arrive on or before the deadline date**. [Sending the practical exam to the Association Management Office delays the grading of the exam and increases shipping costs.] The Certification Board Chairman will provide you with the appropriate shipping address when you are informed of passing the written examination. We recommend using a courier like UPS or Federal Express; if you use the U.S. Postal Service, send the exam by express, certified, or registered mail. MSA is not responsible for damage to examination materials in transit.

5. Submitted examination materials are held confidential, become the property of MSA, and are not returned to the applicant. If one or two grids per tissue are damaged or not usable, grades will be based on the remaining grids; if there are more than two damaged grids per sample or unlabeled or missing material, or other deficiencies, the examination may be returned for re-submission in a later cycle.
Reference Books on Transmission Electron Microscopy


CHAPTER 24

Infection at the Cellular Level

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Abstract

Fine structural analysis of the infection process is indispensable for understanding the relation between microorganisms and host cells. This chapter focuses on standard techniques for transmission as well as scanning electron microscopy that will be of benefit even to researchers new to the field.

I. Introduction

After the “descriptive” era (1960–1970) of electron microscopy, the use of EM in the study of microorganisms declined. During the last 15 years however, tools were developed to modify tissues and cultured cells as well as pathogens on a molecular
basis either by expressing additional genes or by silencing genes by knock-out techniques (somatic gene deletion, interfering RNA). Thus, precisely defined alterations were introduced, and the interest in fine structural analysis of these genetically modified systems gave rise to a new era of electron microscopy. The expression of proteins with fluorescent tags in living cells allows the correlation between live cell imaging and subsequent fine structure analysis of the same cell, this time identifying the transfected proteins on an EM level either by immunogold techniques, or more directly, by photoconversion inducing an electron-dense precipitate.

In this chapter, we present preparation methods that will enable workers from other research areas to process their samples and have them analyzed in the electron microscope. Most of the methods we will present can be successfully used without great manual or technical skills provided that access to the usual EM periphery (ultramicrotome, critical point dryer, sputter coater, etc.) is given.

Although a more detailed and probably more live-like appearance of microorganisms can be obtained by specialized techniques like cryo-EM of vitreous sections (CEMOVIS; Al-Amoudi et al., 2004), these methods are far beyond reach of a conventional laboratory and are not topics of this chapter.

II. Methods and Materials

Choosing which method is most suitable for the fine structure analysis of a microorganism depends on the available source. The source could be

- isolated microorganisms from cultures or clinical samples
- infected primary cells or cell cultures, or
- tissue from infected animals or humans

For isolated microbes, suitable methods range from negative staining techniques, ultrathin sections to scanning electron microscopy (SEM). Cell cultures are most often analyzed with transmission electron microscopy (TEM) or SEM depending on the underlying question, and most tissue samples are embedded and viewed using a TEM. All techniques can be combined with immunodetection methods. On the following pages we will give an overview on the techniques we use to study infection at the cellular level.

A. Negative Staining of Isolated Bacteria

Bacteria are harvested from plates or liquid cultures, washed twice in PBS, and fixed using 1–2.5% glutaraldehyde/PBS for best preservation of fine structure, or 2–4% PFA dissolved in PBS for immunodetection. If the fine structure is not sufficient, glutaraldehyde at concentrations between 0.05% and 0.1% can be added (Hayat, 1981). The following sample preparation can also be used for fixed suspensions of fractions of bacterial or cellular components or enriched suspensions of viruses, particles, etc.
1. Sample Adhesion to Carbon-Coated Formvar Films

Small droplets (20–50 μl) of the bacterial suspensions are placed on Parafilm® in a humid chamber (plastic boxes with a lid having wet filter paper along the edges). Grids (200 mesh) with carbon-coated formvar or pioloform films are placed with the coated side on the droplets. The grids have to be glow discharged (Dubochet et al., 1971) to ensure even wetting of the surface and to assure binding of bacteria to the grids which normally takes a couple of minutes. Clinical samples often do not contain high amount of particles, and are normally contaminated with other materials, so several washing cycles or gradient purification may be required before negative staining.

If the desired density of particles on the EM-grid is not reached by adhesion to the floating grid, the suspension can be enriched by centrifugation prior to the adhesion step. Improved adhesion to the grid can also be achieved by carefully sticking the grids on Parafilm® in a humid chamber with the carbon-film side facing upwards and by placing droplets of 3–4 μl of the suspension on the film for up to 1 h. It is important for the following steps that only the filmed side of the grid is wetted. This will ensure that the grids will float on drops in all the following steps, which prevents contamination of the noncoated side of the grid as well as chemical reactions of the copper with other reagents.

2. Negative Stain

For the conventional negative stain the grids are removed from the specimen drops, washed five times on drops of distilled water to remove salts, and then placed on drops of the aqueous solution of heavy metal salts (2% uranyl acetate, 2% phosphotungstic acid, or 3% ammonium molybdate) for 10 s–2 min (Harris and Horne, 1994). The concentration of the contrasting solution is not critical, but it is advisable to centrifuge the contrasting solution for some minutes at high speed to sediment any precipitates before use. After that, the grids are removed from the droplets with a fine forceps, and the negative stain is partly removed by holding the coated surface of the grid against filter paper. The contrast achieved depends on the thickness of the remaining negative contrast solution and varies with the angle between grid and filter paper: a small angle will result in a thin layer and lower contrast. This is especially desirable if isolated bacterial compounds like flagella are to be analyzed. After air drying the grids can be examined in TEM.

If complete bacteria are to be analyzed, the negative stain density can be adequate for small structures like flagella, but too dense to show details of the bacterial cells (see Fig. 1A). In this case, a series of specimens contrasted under different conditions has to be prepared. Negative contrast is a good way to give information on purity of bacterial fractionations prepared for nonmicroscopic techniques like protein gel electrophoresis (see Fig. 1B).
3. Immunogold Labeling Combined with Negative Staining

For antigen localization studies negative staining can easily be combined with immunogold detection. This method is quick and gives nice results especially when epitopes on the surface of thin structures like flagellae, pili, etc. are to be examined. Following attachment to the coated side, the grids are washed three times on drops of PBS and transferred to a blocking solution (1% BSA, 0.02 M glycine, 10% cold water fish gelatine in PBS). After blocking for 30 min, grids are transferred to the primary antibody (1–10 μg/ml in blocking buffer) and incubated for 30–60 min. Following washes on PBS drops (6 × 2 min), the grids are incubated for 30 min with secondary antibodies coupled to gold colloids (in blocking buffer).

If correlative studies including samples with fluorescent immunostains are planned it is a good idea to use gold coupled secondary antibodies of the same source as the fluorescent coupled secondary antibody. The choice of colloid diameter depends on the planned magnification: while 6 nm colloids require a magnification of at least 20,000×, 18 nm colloids are already visible in low magnification overviews. As shown earlier, the particle density of the label is higher with smaller colloid diameter (Fig. 2B and C.) (Slot and Geuze, 1981). The specimens are incubated with antibodies in a moist chamber at 37 °C. After washing (6 × 2 min PBS, 5 × 2 min distilled water), the negative staining is performed as mentioned previously. A low contrast is desirable to clearly depict the gold colloids.

4. Labeling with Quantum Dots

Semiconductor nanocrystals, commercially available as Quantum Dots or Qdots, play an increasing role as fluorescent probes in biomedical research (Michalet et al., 2005). Owing to their metal core they appear rather electron
dense, and due to their uniform size they can also be used as immunoprobes for TEM structure analysis. They give good labeling densities and are easily visualized in TEM images if a low contrast of the specimen is achieved (see Fig. 2). After incubation of specimens with the primary antibodies and washing, the samples are incubated with a suspension of Quantum Dots coated with secondary antibody. After washing, a low contrast negative stain is applied as mentioned earlier. Note that the electron microscope may reveal even more Quantum Dots than the light microscope in a comparable staining experiment because a subfraction of a given Quantum Dot preparation is sometimes in a permanent dark state and thus does not emit fluorescence light (Yao et al., 2005).

The evaluation of staining with Quantum Dots at high magnifications on pioloform-carbon-coated grids may be hindered by the structure of the film, which may prevent detection of all Quantum Dots owing to their low contrast compared to gold particles. Qdots are better visible, if the pioloform film is partially or in total dissolved in a way that only the structureless carbon layer remains (Fig. 3).

Coat grids with a thin layer of pioloform. Coat the other side of the grid with 20–30 nm carbon. Remove the film by dipping the grid carefully into chloroform for some seconds (Pontefract and Bergeron, 1981). To preserve the beam stability of the carbon layer we recommend not to remove the film completely, but to induce holes. Figure 3 shows that Quantum Dots are better visible in areas where the pioloform film is missing, and obscured in areas with undissolved film layer.

Fig. 2  Correlating fluorescence and negative staining of bacterial flagellae with polyclonal antibodies. (A) Light microscopic fluorescence image of Salmonella typhimurium labeled using secondary antibodies coupled to Quantum Dots. Scale bar: 2.5 μm; (B, C) negative contrast TEM images: (B) labeled with the same primary antibody, detected with secondary antibody coupled to 6 nm gold particles. Scale bar: 100 nm; (C) labeled simultaneously as before with Quantum Dots (λ: 565 nm, arrowheads) and with 12 nm gold particles (arrows). The labeling density achieved with Quantum Dots is significantly greater. Scale bar: 100 nm.
5. Correlative Light- and Transmission-Electron Microscopy of Negative Stained Samples

Before visualization of a structure by negative staining techniques in the TEM it may be interesting to study it at light-microscopy level using fluorescent dyes or phase contrast to screen a number of samples quickly or to find a suitable specimen detail before TEM analysis. Often, correlative microscopy combining light and electron microscopy gives a more complete picture than using TEM alone. It can be useful to identify certain fine-structural phenotypes with patterns occurring at light-microscopy level and vice versa. Host cell–pathogen interactions are one example. The appearance of mammalian cells in a negative contrasted TEM image reveals little more than the cytoskeleton due to the harsh extracting and air drying conditions during the staining procedure, but extracellular structures can be nicely preserved.

Light microscopic examination of adherent cells and/or bacteria can be carried out on the EM-grid after adhesion and before the negative staining step. For this, cells are cultivated on the carbon surface of a film-coated copper grid. Again it is important for later processing to keep the back surface of the grid dry. This can be achieved by applying very small volumes of media on a grid stuck to Parafilm®. Another method is to place drops of 10 μl of cell suspension in medium on the wells of a 12-well teflon-coated diagnostic slide and to invert the slide after applying the TEM-grids to the drops so that the cells settle on the grid, suspended under the hanging droplet. Note that the grids will always float to the rim of the droplet when the slide is inverted. The droplets should be small enough to have the grid suspended nearly parallel to the slide under the medium. The slide can be kept in

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**Fig. 3** *Salmonella* flagellae stained with polyclonal antibody rabbit-anti-*salmonella*, secondary antibodies goat-anti-rabbit Qdot 565 together with goat anti-rabbit, conjugated with 12 nm gold. Scale bar: 100 nm.
a moist chamber supported on Plasticine® beads at 37 °C to allow for cell adhesion and interaction. It is good to start with around $10^3$ cells per grid. Fixation can be carried out after the slide has been turned upward again by keeping the grids floating on the droplets and carefully adding an appropriate amount of fixative stock solution.

After fixation and, depending on the experiment, extraction, permeabilizing and fluorescent staining of the grids, the grids can be stuck to a large glass-coverslip with a small drop of glycerol with the carbon-film side facing the glycerol. Excess glycerol should be removed, so that the layer of glycerol is thin enough to view the cells through the other side of the glass with an immersion lens of a light microscope. After acquiring light microscopic images using phase contrast or fluorescence illumination the grids are brought back into floating condition by carefully adding distilled water to the glycerol layer between the glass and the grid. Alternatively, if light microscopic images of lower magnification are sufficient, the grids can be viewed and imaged floating on a culture medium droplet on the 12-well-slide with a long working distance lens. Note that Qdots may not fluoresce in this setting, possibly because of their sensitivity to quenching effects. The negative staining procedure is carried out as before starting with the five washing steps on drops of distilled water.

Figure 4 shows human neutrophil granulocytes infected with *Staphylococcus aureus*. After stimulation, the cells flatten down (Fig. 4A) and release neutrophil extracellular traps (NETs; Brinkmann et al., 2004). The fine structure can be studied by negative contrast in TEM (Fig. 4C and D).

**B. Resin Embedding of Bacteria-Infected Cell Cultures**

For resin embedding, the microbe to be analyzed directs the choice of the resin. Bacteria with a very hydrophobic cell wall, like mycobacteria, cannot successfully be embedded with standard resins like the Epon derivative Polybed (Fig. 6A). We compared the ultra structure of different bacteria embedded into different resins as well as ultra-thin cryosections.

1. Fixing Specimens

For the analysis of infection at the cellular level, in most cases we fix by adding the desired amount of fixative stock solution into the warm tissue culture medium. This ensures that no alterations of the cell culture due to media changes are introduced. Bacteria on the surface of the cells are not washed away, but will be kept in place even if they are not firmly attached to the cell surface. We then keep cell culture plates at RT for two hours after which period even pathogenic bacteria can be processed in a regular S1 lab.

To maintain physical integrity of the cells, we do not scrape them off the plate after fixation. This induces membrane breaks, distortion of plasma fine structure and regularly leads to loss of the basal domains of the cells. Especially analysis of
the membrane integrity of i.e., phagosomes is severely aggravated if scraped cells are used. Instead, we leave the cells on the plate during postfixation, contrasting, and dehydration. We usually use styrene as intermediate step before embedding into resin. Styrene dissolves the tissue culture plates, so if timed correctly, the cells become detached without physical damage. We use a shaker to move the styrene over the cells. The time necessary to detach the cells varies with the cell type between 1 and 10 min; confluent epithelial layers remain on the plastic for a long time and then detach as multicellular aggregates. It can be useful to cut an X into the layer with a scalpel before styrene is added to allow a faster detachment. If the

Fig. 4 Correlation of whole mounts of cells cultivated on filmed grids. Human neutrophils and *Staphylococcus aureus* on a film-coated EM-grid, (A, B) light microscopic images of fixed cells and bacteria, detection of DNA with SYTOX Green, (C) negative contrast TEM image of the same mesh, (D) negative stain image of *S. aureus* and neutrophil extracellular traps (*Brinkmann et al., 2004*) from the same experiment. Scale bars: (A–C) 10 μm, (D) 1 μm.
24. Infection at the Cellular Level

Styrene is on the plates for too long, the bottom of the plate dissolves into a smear that contaminates the cell preparation. Should this happen, the resulting suspension should be transferred to a 15 ml bluecap that is filled with styrene. After vortexing and centrifugation, the cells separate from the dissolved plastic and can be pelleted. The plastic-contaminated supernatant is removed carefully, and the sediment is then resuspended in fresh styrene and embedded using styrene-resin mixtures. A 1:2 mixture is left in an open cylindrical glass vessel overnight to allow complete infiltration and increasing resin concentration due to partial evaporation of the styrene. The next morning, three changes using undiluted resin finish the embedding. With increasing resin concentration, the cells do not sediment spontaneously. Thus, to ensure that during replacement the supernatant no cellular material gets lost, the specimens are transferred to Eppendorf® tubes and centrifuged for about 10 min in a swing out rotor at about 350 × g. With the resin changes, the cells become resuspended. After the final change, the cells are sedimented, a paper tag with the specimen identification is inserted into the cap, and the specimens are polymerized for 1–2 days. For cultured cells, we prefer this method to flat embedding, since the concentration of cells remains high, while during flat embedding, cell clusters tend to float apart thus reducing the number of cells per section.

2. Staining of Semithin Sections for Light Microscopic Overviews

After polymerization, semithin (200–500 μm) sections are prepared with a histology diamond knife. Transfer them to a drop of distilled water on a microscope slide with an eyelash or a grid, and let the drop dry on a hot plate at about 80 °C. The sections are then stained with filtered solution of 1% toluidine blue in 1% borax on the hot plate. The drop of staining solution remains on the sections until it starts to dry out at the edges. Before crystals are formed, the slide is washed in tap water and transferred to distilled water until no further stain washes out and the water remains clear. The slide can then be mounted and analyzed with a light microscope. If necessary, the block can be retrimmed to the area of highest interest, before ultra-thin sections are prepared. Figure 5 shows the comparison of light- and electron microscopic images of macrophages infected with mycobacteria.

For fine structure analysis, we routinely use uranyl acetate block staining, so ultra-thin sections normally carry enough contrast to be photographed with a digital camera, which sets the boundaries of the image histogram in a way that areas of highest brightness will be white while pixels of lowest brightness will be black. Thus, a high-contrast image is created, which can be evaluated without further processing. Nonetheless, for print quality images further contrasting of the section (e.g., with lead citrate) is indispensable to avoid the introduction of background noise that is created by stretching the histogram boundaries on a weakly contrasted image. Furthermore it is uncomfortable to evaluate a weakly or noncontrasted section on the TEM screen.
3. Comparison of Different Embedding Media

Polybed 812 has displaced classical Epon 812 and is now the probably most widely used resin. Completed by NMA (Nadic Methyl Anhydride) as epoxy resin, DDSA (Dodecenylsuccinic Anhydride) as hardener, and DMP-30 (2,4,6 Tris (dimethylaminomethyl)phenol as accelerator it gives hard blocks, easy to section and stable under the electron beam even under high emission current (~35 μA). Of course, the ratio of components can be varied to alter the hardness of the blocks. Although Polybed 812 has many advantages, it has limited value for preparations of microorganisms with waxy cell walls (mycobacteria, Fig. 6A) or for high density organisms like Staphylococcus (Fig. 6E) or for elementary bodies of Chlamydia (Fig. 6M). Polybed will not penetrate the particles sufficiently; the resulting sections are instable and may even have holes. For embedding of dense material, the use of media with reduced viscosity is advisable.
“Spurr’s” low-viscosity embedding medium was developed in the late 1960s primarily to meet the needs of botanists for a low-viscosity resin that would more easily penetrate the cell walls. It consists of four components: Based on the

Fig. 6 Comparison of different embedding media. The widely used Polybed 812 will not penetrate efficiently into mycobacteria and staphylococci resulting in holey unstable sections (A, E). More reliable results are obtained with low-viscosity media like Spurr’s (B, F) and Embed-It (C, G). Due to reduced lipid extraction, cryopreparations can reveal more details (D, H). (columns from left to right: Polybed, Spurr’s, Embed-It, Cryopreparation). A–D: Mycobacteria (BCG), E–H: Staphylococcus aureus, I–L: Neisseria meningitides, M–P: Chlamydia trachomatis. Scale bars: 500 nm.
cycloaliphatic diepoxide vinylcyclohexene oxide (VCD), it uses the epoxy resin D.E.R. 736 (diglycidyl ether of polypropylene glycol) as flexibilizer, in addition to NSA and accelerator dimethyloaminoethanol (DMAE). The single components allow to specify the properties of the cured blocks depending on the respective purpose, like rapid polymerization or desired block hardness. “Spurr’s” viscosity of 60 cps leads to a better and quicker penetration of hydrophobic material, such as cell walls of Gram positive bacteria (Fig. 6F) and very dense structures such as spores, elementary bodies of Chlamydiae (Fig. 6N), and yeast cell walls. Its liquidity makes it easy to work with. Specimens sediment quickly, so less material is lost during resin exchange. Spurr resin unfortunately has less than ideal characteristics for ultrathin sectioning. It tends to stick to the cutting edge of the diamond knife and produces folded aggregates of sectioned materials. To resolve this problem antistatic devices can be used during cutting to obtain a consistent and steadily produced ribbon of sections. Another disadvantage is the instability of Spurr resin in the electron beam. It rips very quickly on exposure to the beam and is even more sensitive after lead citrate staining. To overcome this problem, the sections can be relatively stabilised by starting observations at low magnification (1000×) and low emission current (ca. 15 μA).

Embed-It™ (Polysciences, Inc.) was created as an easy-to-mix polymer of low-viscosity (65 cps) and also to avoid inconsistency between blocks. The “Spurr’s” derivative consists of two nonhazardous components, its viscosity is similar to Spurr’s, while the stability of the sections in the TEM is comparable to Polybed.

If the Polybed mixture does not penetrate samples sufficiently, it may help to prolong incubation times before changing to a different resin. A last incubation step overnight in 100% resin will produce better results, as will additional steps using different intermediate/resin ratios (3:1, 2:1, 1:1, 1:1.5, 1:2, 1:3). Changing the ratios too quickly may cause excessive shrinking and lead to holes in the specimen at, e.g., the space of a phagosome, between the phagosomal membrane and enclosed bacteria.

The comparison of the different resins shows that although all three are suitable for gram negative bacteria (e.g. Neisseria, Fig. 6I–K), Polybed is recommended because of easier section handling. Besides that, it is less toxic than VCD. Very dense structures such as elementary bodies of Chlamydiae, spores, gram positive bacteria (e.g., Staphylococcus) are better and quicker penetrated by Spurr’s resin, although even Polybed works sufficiently well in most cases. Polybed embedding is however completely unsuitable for highly hydrophobic structures such as the waxy cell walls of mycobacteria (Fig. 6A). These structures need resins like Spurr’s (Fig. 6B and F) or Embed-It (Fig. 6C and G). The latter is easier to handle than Spurr’s and is stable under the electron beam; however it cannot totally replace Spurr’s resin, since it needs longer incubation times due to its higher viscosity. Spurr’s is definitely the resin of choice for quick embeddings of dense or hydrophobic structures. It is noticeable that Spurr’s produces a slightly weaker contrast than Polybed or Embed-It sections. Contrast can be improved by longer incubation times of lead citrate but this may produce artefacts due to lead precipitation.
This is the standard embedding protocol for glutaraldehyde-fixed tissue culture specimens:

- 10’ three washings with PBS
- 60’ 0.5% osmium tetroxide in distilled water
- 10’ 4 washings with distilled water
- 60’ 0.1% tannin in 20 mM HEPES buffer
- 10’ four washings with 1% Na₂SO₄ in 20 mM HEPES
- 10’ four washings with distilled water
- 60’ 2% uranyl acetate in distilled water
- 5’ each in 30/50/70/80/90% ethanol
- 3 × 5’ 100% ethanol
- 30’ styrene, over night 1:2 styrene/resin
- 60’ 1:3 styrene/resin
- 3 × 60’ freshly prepared resin over night resin 1–2 days embedding at 60 °C
  (don’t exceed 24 h for “Spurr’s”)

**C. Resin Embedding of Infected Tissue**

For embedding of infected tissue, it is essential to keep the size of the tissue specimen small (less than 2 mm × 2 mm × 2 mm). It is advisable to select the areas of an infected organ that will be embedded with a stereo microscope and only dissect parts that promise to be highly interesting, for the EM analysis does not allow screening of larger tissue sections. Because fixative and solvent penetration takes place over considerably larger distances in tissue, incubation times should be at least five times longer than for cell culture specimens.

It is possible to reanalyze tissue that has been embedded into paraffin for routine histology. Of course, fixation in formalin as well as heating to 65 °C are detrimental for tissue fine structure, but in some instances the reembedding into resin and subsequent EM analysis can be helpful. An advantage of this method is that particularly interesting tissue areas can be identified in sections using histological staining or light-microscopy immunodetection methods. Small cubes including these areas are then cut out of the paraffin block using a scalpel, rehydrated slowly, postfixed with glutaraldehyde and osmium tetroxide and reembedded using the standard embedding protocol. Care must be taken to remove the paraffin wax completely (Gonzalez-Angulo et al., 1978), otherwise penetration with the resin will not be successful.

After embedding, tissue blocks are transferred to cavities of flat embedding forms, laser printed identification tags are inserted and the cavity is filled with fresh resin. The sample is aligned in parallel to the tip of the cavity to ensure quick trimming and the option to section the entire tissue block surface.
D. Rapid Processing for TEM

It can be desirable to shorten the preparation of TEM specimens, e.g., of diagnostic samples. Resin embedding using microwave-assisted tissue processing reduces embedding time down to 4–5 h (Schroeder et al., 2006); alternatively cryomethods can be used which can provide better ultrastructure (compare Fig. 6I–K to Fig. 6L) since less material gets extracted compared to treatment with organic solvents (Korn and Weisman, 1966).

E. Preembedding Immunodetection Methods

Conventional resin techniques are compatible with immunodetection if the antibodies are employed before embedding. If the antigens of interest are extracellular, antibodies can be incubated with living cells on ice (to limit internalization of antibody complexes) or with cells fixed with formaldehyde (2–4% in PBS) for 30–120 min. Cells are then washed (living cells with ice-cold PBS, and then fixed with PFA), before incubation with the secondary antibody which is coupled to gold colloids (30–60 min at RT). After washing, cells are postfixed using 2.5% glutaraldehyde in PBS, and embedded conventionally. This method results in good ultrastructure combined with excellent detection of surface antigens.

Intracellular antigens are only accessible after limited permeabilization. The choice and concentration of the detergent depends on the localization of the antigen, i.e., how many membrane systems have to be crossed by the antibody before it can bind to its antigen. Our first choice is saponin at concentrations around 0.1%. Owing to the permeabilization, loss of ultrastructure cannot be avoided. To assure good penetration, antibody fragments (Fab2) should be employed, and the diameter of the gold should be less than 10 nm. Alternatively, ultra small gold (1 nm diameter or less) can be used which has to be silver-intensified before embedding (Danscher, 1981). In this case, osmium tetroxide should be omitted. Quantum Dots can be used in a similar way as ultra small gold probes. They offer the advantage that the staining can be analyzed on the light microscopic level, before the Quantum Dots are silver enhanced and processed for electron microscopy (Stoltenberg et al., 2007).

F. Immunodetection Using Ultrathin Cryosections

For more than thirty years, ultra-thin sections of frozen material have been used for immunodetection (Tokuyasu, 1973, 1978). Although in the meantime antibody labeling methods with specialized resin techniques have been developed, cryosections are still widely used since the antigen preservation in weakly fixed frozen material is superior to resin techniques. Furthermore, extraction of cytoplasmic material is reduced. The structural preservation in ultra-thin cryosections is often of poorer quality than in resin sections mainly due to the softer fixation methods employed.
Structural preservation and antigen reactivity have to be balanced to obtain optimal results. The contrast in TEM images of cryosections is weaker and less differentiated than in osmicated samples of resin sections. Although fine-structure resolution in resin sections is often superior, EM-sample preparation by cryosectioning takes less time. Also, problems with resin polymerization in bacteria that show a particularly resistant cell wall will not come up under cryosectioning conditions.

Samples of infected cells for cryosectioning can be prepared from adherent cells grown in 6-well culture plates. One sample should consist of at least one confluent well, or preferably two wells (>10⁶ cells). It is important to achieve a high infection rate in order to obtain enough representative cross sections of pathogens in the resulting ultra-thin sections. Cell density and infection can be monitored with an inverted light microscope using phase contrast illumination.

Fixation of the infected cells should take place at incubation temperature to prevent artefacts due to temperature shock. Fixation in PBS or in the growth medium works well in most cases. In some cases further improvement in structural preservation is observed when fixing the cells in cytoskeleton stabilizing buffer (1 mM EGTA, 4% polyethylene glycol 6000 (8000), 100 mM PIPES pH 6.9, (Lindroth et al., 1992)). A combination of 2–4% PFA with 0.05% glutaraldehyde, applied for two hours is a good starting point for many antigens and results in acceptable structure preservation combined with sufficient antigen reactivity. This is discussed in detail in the classic book by Griffith (1993).

Infected cells are harvested with a rubber scraper and suspended in 10% gelatine in PBS at 37 °C and immediately centrifuged to sediment as a pellet. This is excised from the tube after cooling and gelatinizing and cut into smaller pieces fit for infiltration in sucrose solution (2.3 M sucrose in sodium phosphate buffer with 1% PFA, pH 7.4) After infiltrating preferably in a cold room and on a slow inverting shaker overnight the gelatin bits can be mounted on aluminium stubs and frozen in liquid nitrogen for cryosectioning. Sectioning is carried out at −120 °C. Sections are transferred to carbon-coated pioform films on copper or nickel EM-grids and kept section side down on 2% gelatine until immunolabeling and contrasting. For a detailed description of cryosectioning techniques see Chapter 8, this volume.

A basic immunogold labeling of ultra-thin cryosections is carried out as follows: After blocking the sections by floating the grids on drops of blocking buffer (1% BSA, 0.02 M glycine, 10% cold water fish gelatine in PBS) for at least 30 min. Primary antibodies are applied at 1–10 µg/ml diluted in blocking buffer. After incubating for 1 h at 37 °C or over night at 4 °C in a humid chamber, excess antibody is removed by washing 6 × 3 min on drops of PBS. Secondary antibody coated gold colloids are applied for 30 min–1 h at 37 °C diluted in blocking buffer. The labeled grids are washed 6 × in PBS and 5 × in distilled water prior to negative contrasting and embedding in methyl cellulose. For this, the sections are floated on three consecutive drops of 0.2% uranyl acetate and 2% methyl cellulose in distilled water for 2.5 min each before blotting off excess contrasting solution and air drying the grids.
Again, this technique can be correlated with light microscopy. Ribbons of semithin sections can be transferred to glass coverslips together with the pick-up solution. After washing the coverslip on three consecutive drops of PBS, the sections can be stained with a DNA dye and mounted for quick viewing with a fluorescence microscope or used for immunofluorescence staining. The comparison between immunogold labeling and immunofluorescence can help to secure staining specificity if the antigen is only present in small amounts. While intense immunogold staining that correlates with morphology (Fig. 7) is easy to interpret, specificity of a weak staining especially of cytoplasmic antigens can more reliably assessed if correlated with immunofluorescence.

G. Scanning Electron Microscopy

1. Scanning Electron Microscopy of Cultured Cells

For Scanning Electron Microscopy (SEM) analysis, we routinely grow cells on round coverslips (dia. 13 mm). Cells are fixed by adding the fixative stock solution to the medium at 37 °C. If no immunodetection is intended, we use glutaraldehyde at a final concentration of 2.5% for 120 min at room temperature. The fixative is directly added to the cell cultures to avoid loss of material or changes in the pathogen/host interplay. Thus, even fragile structures like NETs (see Fig. 8) can be sufficiently stabilized to survive the consecutive preparation steps.

After several washes with H$_2$O, the specimens are postfixed for 30 min with OsO$_4$ to stabilize membranes. Cells are washed repeatedly with H$_2$O, and then transferred for 30 min to a buffered solution of tannic acid (0.5% in 20 mM HEPES). Repeated treatment with osmium and tannic acid will deposit a

![Fig. 7](image)

**Fig. 7** Detection of mycobacterial antigens on ultrathin cryosections of infected macrophages. The indirect immunogold method using 12 nm gold colloids reveals that mycobacterial antigens are exclusively located inside the phagosome. Scale bar: 500 nm.
conductive layer on the cells which helps minimizing surface charging to a degree that no additional metal layer will be necessary if the specimens are to be analyzed at low acceleration voltage (<1 kV). After the last treatment with osmium, cells are dehydrated using a graded ethanol series. We use molecular sieves to keep the 100%-ethanol water free which requires the filtration of the dry ethanol to remove debris of the molecular sieves. After three dehydration steps using 100% ethanol, cells are transferred to a critical point drying apparatus equipped with a holder for the coverslips, and dried. Since we regularly examine specimens at 20 kV, we coat the surface of the dry cells with 3–5 nm of platinum/carbon. This results in a coating with a finer grain than sputtering them with gold or gold–palladium.

2. Immunodetection Using SEM

For detection of surface antigens, we fix the cells by adding stock solutions of PFA to the warm medium to a final concentration of 4%. After 30 min at room temperature, the specimens are washed with PBS and blocked using 1% BSA in PBS. Primary antibodies are diluted in the same buffer at a concentration between 1 and 10 μg. The coverslips with the cells facing the bottom is placed on 50 μl drops of the antibody solution on parafilm in a humid chamber and incubated at 37 °C for 30–60 min. After repeated washing, the secondary antibody coupled to gold colloids is applied correspondingly. Although the labeling density is lower, we prefer medium-sized gold particles (12–15 nm), because they are more readily identified in the SEM than smaller particles (6 nm or less).
We take material contrast views with the backscattered electron detector (BSE) (Fig. 9A) and topographic images with the secondary electron (SE) detector (Fig. 9B). Even when the specimens are coated with a layer of 2–3 nm platinum/carbon, the gold signal is clearly visible with the BSE detector. Using individual images from both type of detectors normally allows easier identification of gold signal than images created with mixed signal from both detectors at the same time. For presenting both images in an overlay, SE and BSE micrographs can be pseudocolorized using different look-up tables, (e.g., green and red) (Brinkmann et al., 2004).

### III. Overview and Conclusion

In this article we have presented some standard preparations for both TEM and SEM, which will be helpful to infection biologists who would like to get a more precise view on the process of infection. Yet certain caveats have to be kept in mind. Compared with that in light microscopy, in electron microscopy the number of individual cells in a given sample is normally much smaller. Great care has to be taken to assure that an adequate number of individual cells are analyzed to represent the entire sample; thus, it is of great importance to elaborate infection...
parameters that ensure homogenous infection patterns in cell culture or organ samples. It is a good idea to correlate light and electron microscopy to be able to monitor the infection status of the entire sample before concentrating on fine structure analysis of a relatively small number of cells.

If knock-down techniques are used to repress expression of a certain gene, it is necessary to carefully monitor the degree of gene silencing. While primary cells or organs from mice with properly induced KO germline mutations will surely not express the protein under investigation, silencing genes with RNA techniques never repress protein expression entirely. Often, 20–40% of residual expression remains, which may be enough to yield a readout using light microscopic techniques, but is not satisfactory for electron microscopy, which concentrates on a limited number of individual cells. Thus, for EM studies RNA-induced gene silencing is only adequate if a high repression rate (>90%) is accomplished, and the number of analyzed cells is big enough to be statistically significant.

For a researcher new to transmission electron microscopy it is often dissatisfactory that the number of infected cells in a given sample seems to be much smaller than in a corresponding sample processed for light microscopy. It has to be kept in mind that while light microscopy always displays the entire cell and even a single bacterium infecting this cell can easily be visualized, an ultra-thin section of 60 nm only represents about 0.5–1% of the cell, and thus many cell profiles erroneously appear uninfected. This again is an argument for correlative studies.

The multiple facets of electron microscopy offer indispensable tools for studying infection processes. We would like to encourage more biologists to use electron microscopy in their infection models to gain a more precise insight into the biology of infection.

References


CHAPTER 24

Infection at the Cellular Level

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Abstract

I. Introduction
II. Methods and Materials
   A. Negative Staining of Isolated Bacteria
   B. Resin Embedding of Bacteria-Infected Cell Cultures
   C. Resin Embedding of Infected Tissue
   D. Rapid Processing for TEM
   E. Preembedding Immunodetection Methods
   F. Immunodetection Using Ultrathin Cryosections
   G. Scanning Electron Microscopy
III. Overview and Conclusion

References

Abstract

Fine structural analysis of the infection process is indispensable for understanding the relation between microorganisms and host cells. This chapter focuses on standard techniques for transmission as well as scanning electron microscopy that will be of benefit even to researchers new to the field.

I. Introduction

After the “descriptive” era (1960–1970) of electron microscopy, the use of EM in the study of microorganisms declined. During the last 15 years however, tools were developed to modify tissues and cultured cells as well as pathogens on a molecular
basis either by expressing additional genes or by silencing genes by knock-out techniques (somatic gene deletion, interfering RNA). Thus, precisely defined alterations were introduced, and the interest in fine structural analysis of these genetically modified systems gave rise to a new era of electron microscopy. The expression of proteins with fluorescent tags in living cells allows the correlation between live cell imaging and subsequent fine structure analysis of the same cell, this time identifying the transfected proteins on an EM level either by immunogold techniques, or more directly, by photoconversion inducing an electron-dense precipitate.

In this chapter, we present preparation methods that will enable workers from other research areas to process their samples and have them analyzed in the electron microscope. Most of the methods we will present can be successfully used without great manual or technical skills provided that access to the usual EM periphery (ultramicrotome, critical point dryer, sputter coater, etc.) is given.

Although a more detailed and probably more live-like appearance of microorganisms can be obtained by specialized techniques like cryo-EM of vitreous sections (CEMOVIS; Al-Amoudi et al., 2004), these methods are far beyond reach of a conventional laboratory and are not topics of this chapter.

II. Methods and Materials

Choosing which method is most suitable for the fine structure analysis of a microorganism depends on the available source. The source could be

- isolated microorganisms from cultures or clinical samples
- infected primary cells or cell cultures, or
- tissue from infected animals or humans

For isolated microbes, suitable methods range from negative staining techniques, ultrathin sections to scanning electron microscopy (SEM). Cell cultures are most often analyzed with transmission electron microscopy (TEM) or SEM depending on the underlying question, and most tissue samples are embedded and viewed using a TEM. All techniques can be combined with immunodetection methods. On the following pages we will give an overview on the techniques we use to study infection at the cellular level.

A. Negative Staining of Isolated Bacteria

Bacteria are harvested from plates or liquid cultures, washed twice in PBS, and fixed using 1–2.5% glutaraldehyde/PBS for best preservation of fine structure, or 2–4% PFA dissolved in PBS for immunodetection. If the fine structure is not sufficient, glutaraldehyde at concentrations between 0.05% and 0.1% can be added (Hayat, 1981). The following sample preparation can also be used for fixed suspensions of fractions of bacterial or cellular components or enriched suspensions of viruses, particles, etc.
1. Sample Adhesion to Carbon-Coated Formvar Films

Small droplets (20–50 μl) of the bacterial suspensions are placed on Parafilm® in a humid chamber (plastic boxes with a lid having wet filter paper along the edges). Grids (200 mesh) with carbon-coated formvar or pioloform films are placed with the coated side on the droplets. The grids have to be glow discharged (Dubochet et al., 1971) to ensure even wetting of the surface and to assure binding of bacteria to the grids which normally takes a couple of minutes. Clinical samples often do not contain high amount of particles, and are normally contaminated with other materials, so several washing cycles or gradient purification may be required before negative staining.

If the desired density of particles on the EM-grid is not reached by adhesion to the floating grid, the suspension can be enriched by centrifugation prior to the adhesion step. Improved adhesion to the grid can also be achieved by carefully sticking the grids on Parafilm® in a humid chamber with the carbon-film side facing upwards and by placing droplets of 3–4 μl of the suspension on the film for up to 1 h. It is important for the following steps that only the filmed side of the grid is wetted. This will ensure that the grids will float on drops in all the following steps, which prevents contamination of the noncoated side of the grid as well as chemical reactions of the copper with other reagents.

2. Negative Stain

For the conventional negative stain the grids are removed from the specimen drops, washed five times on drops of distilled water to remove salts, and then placed on drops of the aqueous solution of heavy metal salts (2% uranyl acetate, 2% phosphotungstic acid, or 3% ammonium molybdate) for 10 s–2 min (Harris and Horne, 1994). The concentration of the contrasting solution is not critical, but it is advisable to centrifuge the contrasting solution for some minutes at high speed to sediment any precipitates before use. After that, the grids are removed from the droplets with a fine forceps, and the negative stain is partly removed by holding the coated surface of the grid against filter paper. The contrast achieved depends on the thickness of the remaining negative contrast solution and varies with the angle between grid and filter paper: a small angle will result in a thin layer and lower contrast. This is especially desirable if isolated bacterial compounds like flagella are to be analyzed. After air drying the grids can be examined in TEM.

If complete bacteria are to be analyzed, the negative stain density can be adequate for small structures like flagella, but too dense to show details of the bacterial cells (see Fig. 1A). In this case, a series of specimens contrasted under different conditions has to be prepared. Negative contrast is a good way to give information on purity of bacterial fractionations prepared for nonmicroscopic techniques like protein gel electrophoresis (see Fig. 1B).
3. Immunogold Labeling Combined with Negative Staining

For antigen localization studies negative staining can easily be combined with immunogold detection. This method is quick and gives nice results especially when epitopes on the surface of thin structures like flagellae, pili, etc. are to be examined. Following attachment to the coated side, the grids are washed three times on drops of PBS and transferred to a blocking solution (1% BSA, 0.02 M glycine, 10% cold water fish gelatine in PBS). After blocking for 30 min, grids are transferred to the primary antibody (1–10 μg/ml in blocking buffer) and incubated for 30–60 min. Following washes on PBS drops (6 × 2 min), the grids are incubated for 30 min with secondary antibodies coupled to gold colloids (in blocking buffer).

If correlative studies including samples with fluorescent immunostains are planned it is a good idea to use gold coupled secondary antibodies of the same source as the fluorescent coupled secondary antibody. The choice of colloid diameter depends on the planned magnification: while 6 nm colloids require a magnification of at least 20,000×, 18 nm colloids are already visible in low magnification overviews. As shown earlier, the particle density of the label is higher with smaller colloid diameter (Fig. 2B and C.) (Slot and Geuze, 1981). The specimens are incubated with antibodies in a moist chamber at 37°C. After washing (6 × 2 min PBS, 5 × 2 min distilled water), the negative staining is performed as mentioned previously. A low contrast is desirable to clearly depict the gold colloids.

4. Labeling with Quantum Dots

Semiconductor nanocrystals, commercially available as Quantum Dots or Qdots, play an increasing role as fluorescent probes in biomedical research (Michalet et al., 2005). Owing to their metal core they appear rather electron
dense, and due to their uniform size they can also be used as immunoprobes for TEM structure analysis. They give good labeling densities and are easily visualized in TEM images if a low contrast of the specimen is achieved (see Fig. 2). After incubation of specimens with the primary antibodies and washing, the samples are incubated with a suspension of Quantum Dots coated with secondary antibody. After washing, a low contrast negative stain is applied as mentioned earlier. Note that the electron microscope may reveal even more Quantum Dots than the light microscope in a comparable staining experiment because a subfraction of a given Quantum Dot preparation is sometimes in a permanent dark state and thus does not emit fluorescence light (Yao et al., 2005).

The evaluation of staining with Quantum Dots at high magnifications on pioloform-carbon-coated grids may be hindered by the structure of the film, which may prevent detection of all Quantum Dots owing to their low contrast compared to gold particles. Qdots are better visible, if the pioloform film is partially or in total dissolved in a way that only the structureless carbon layer remains (Fig. 3).

Coat grids with a thin layer of pioloform. Coat the other side of the grid with 20–30 nm carbon. Remove the film by dipping the grid carefully into chloroform for some seconds (Pontefract and Bergeron, 1981). To preserve the beam stability of the carbon layer we recommend not to remove the film completely, but to induce holes. Figure 3 shows that Quantum Dots are better visible in areas where the pioloform film is missing, and obscured in areas with undissolved film layer.

Fig. 2 Correlating fluorescence and negative staining of bacterial flagellae with polyclonal antibodies. (A) Light microscopic fluorescence image of Salmonella typhimurium labeled using secondary antibodies coupled to Quantum Dots. Scale bar: 2.5 µm; (B, C) negative contrast TEM images: (B) labeled with the same primary antibody, detected with secondary antibody coupled to 6 nm gold particles. Scale bar: 100 nm; (C) labeled simultaneously as before with Quantum Dots (λ: 565 nm, arrowheads) and with 12 nm gold particles (arrows). The labeling density achieved with Quantum Dots is significantly greater. Scale bar: 100 nm.
5. Correlative Light- and Transmission-Electron Microscopy of Negative Stained Samples

Before visualization of a structure by negative staining techniques in the TEM it may be interesting to study it at light-microscopy level using fluorescent dyes or phase contrast to screen a number of samples quickly or to find a suitable specimen detail before TEM analysis. Often, correlative microscopy combining light and electron microscopy gives a more complete picture than using TEM alone. It can be useful to identify certain fine-structural phenotypes with patterns occurring at light-microscopy level and vice versa. Host cell–pathogen interactions are one example. The appearance of mammalian cells in a negative contrasted TEM image reveals little more than the cytoskeleton due to the harsh extracting and air drying conditions during the staining procedure, but extracellular structures can be nicely preserved.

Light microscopic examination of adherent cells and/or bacteria can be carried out on the EM-grid after adhesion and before the negative staining step. For this, cells are cultivated on the carbon surface of a film-coated copper grid. Again it is important for later processing to keep the back surface of the grid dry. This can be achieved by applying very small volumes of media on a grid stuck to Parafilm®. Another method is to place drops of 10 μl of cell suspension in medium on the wells of a 12-well teflon-coated diagnostic slide and to invert the slide after applying the TEM-grids to the drops so that the cells settle on the grid, suspended under the hanging droplet. Note that the grids will always float to the rim of the droplet when the slide is inverted. The droplets should be small enough to have the grid suspended nearly parallel to the slide under the medium. The slide can be kept in

![Fig. 3](Image)

*Salmonella* flagellae stained with polyclonal antibody rabbit-anti-*salmonella*, secondary antibodies goat-anti-rabbit Qdot 565 together with goat anti-rabbit, conjugated with 12 nm gold. Scale bar: 100 nm.
a moist chamber supported on Plasticine® beads at 37 °C to allow for cell adhesion and interaction. It is good to start with around $10^3$ cells per grid. Fixation can be carried out after the slide has been turned upward again by keeping the grids floating on the droplets and carefully adding an appropriate amount of fixative stock solution.

After fixation and, depending on the experiment, extraction, permeabilizing and fluorescent staining of the grids, the grids can be stuck to a large glass-coverslip with a small drop of glycerol with the carbon-film side facing the glycerol. Excess glycerol should be removed, so that the layer of glycerol is thin enough to view the cells through the other side of the glass with an immersion lens of a light microscope. After acquiring light microscopic images using phase contrast or fluorescence illumination the grids are brought back into floating condition by carefully adding distilled water to the glycerol layer between the glass and the grid. Alternatively, if light microscopic images of lower magnification are sufficient, the grids can be viewed and imaged floating on a culture medium droplet on the 12-well-slide with a long working distance lens. Note that Qdots may not fluoresce in this setting, possibly because of their sensitivity to quenching effects. The negative staining procedure is carried out as before starting with the five washing steps on drops of distilled water.

Figure 4 shows human neutrophil granulocytes infected with *Staphylococcus aureus*. After stimulation, the cells flatten down (Fig. 4A) and release neutrophil extracellular traps (NETs; Brinkmann *et al.*, 2004). The fine structure can be studied by negative contrast in TEM (Fig. 4C and D).

**B. Resin Embedding of Bacteria-Infected Cell Cultures**

For resin embedding, the microbe to be analyzed directs the choice of the resin. Bacteria with a very hydrophobic cell wall, like mycobacteria, cannot successfully be embedded with standard resins like the Epon derivative Polybed (Fig. 6A). We compared the ultra structure of different bacteria embedded into different resins as well as ultra-thin cryosections.

1. **Fixing Specimens**

For the analysis of infection at the cellular level, in most cases we fix by adding the desired amount of fixative stock solution into the warm tissue culture medium. This ensures that no alterations of the cell culture due to media changes are introduced. Bacteria on the surface of the cells are not washed away, but will be kept in place even if they are not firmly attached to the cell surface. We then keep cell culture plates at RT for two hours after which period even pathogenic bacteria can be processed in a regular S1 lab.

To maintain physical integrity of the cells, we do not scrape them off the plate after fixation. This induces membrane breaks, distortion of plasma fine structure and regularly leads to loss of the basal domains of the cells. Especially analysis of
the membrane integrity of i.e., phagosomes is severely aggravated if scraped cells are used. Instead, we leave the cells on the plate during postfixation, contrasting, and dehydration. We usually use styrene as intermediate step before embedding into resin. Styrene dissolves the tissue culture plates, so if timed correctly, the cells become detached without physical damage. We use a shaker to move the styrene over the cells. The time necessary to detach the cells varies with the cell type between 1 and 10 min; confluent epithelial layers remain on the plastic for a long time and then detach as multicellular aggregates. It can be useful to cut an X into the layer with a scalpel before styrene is added to allow a faster detachment. If the

Fig. 4 Correlation of whole mounts of cells cultivated on filmed grids. Human neutrophils and *Staphylococcus aureus* on a film-coated EM-grid, (A, B) light microscopic images of fixed cells and bacteria, detection of DNA with SYTOX Green, (C) negative contrast TEM image of the same mesh, (D) negative stain image of *S. aureus* and neutrophil extracellular traps (Brinkmann et al., 2004) from the same experiment. Scale bars: (A–C) 10 μm, (D) 1 μm.
styrene is on the plates for too long, the bottom of the plate dissolves into a smear that contaminates the cell preparation. Should this happen, the resulting suspension should be transferred to a 15 ml bluecap that is filled with styrene. After vortexing and centrifugation, the cells separate from the dissolved plastic and can be pelleted. The plastic-contaminated supernatant is removed carefully, and the sediment is then resuspended in fresh styrene and embedded using styrene-resin mixtures. A 1:2 mixture is left in an open cylindrical glass vessel overnight to allow complete infiltration and increasing resin concentration due to partial evaporation of the styrene. The next morning, three changes using undiluted resin finish the embedding. With increasing resin concentration, the cells do not sediment spontaneously. Thus, to ensure that during replacement the supernatant no cellular material gets lost, the specimens are transferred to Eppendorf® tubes and centrifuged for about 10 min in a swing out rotor at about $350 \times g$. With the resin changes, the cells become resuspended. After the final change, the cells are sedimented, a paper tag with the specimen identification is inserted into the cap, and the specimens are polymerized for 1–2 days. For cultured cells, we prefer this method to flat embedding, since the concentration of cells remains high, while during flat embedding, cell clusters tend to float apart thus reducing the number of cells per section.

2. Staining of Semithin Sections for Light Microscopic Overviews

After polymerization, semithin (200–500 µm) sections are prepared with a histology diamond knife. Transfer them to a drop of distilled water on a microscope slide with an eyelash or a grid, and let the drop dry on a hot plate at about $80^\circ C$. The sections are then stained with filtered solution of 1% toluidine blue in 1% borax on the hot plate. The drop of staining solution remains on the sections until it starts to dry out at the edges. Before crystals are formed, the slide is washed in tap water and transferred to distilled water until no further stain washes out and the water remains clear. The slide can then be mounted and analyzed with a light microscope. If necessary, the block can be retrimmed to the area of highest interest, before ultra-thin sections are prepared. Figure 5 shows the comparison of light- and electron microscopic images of macrophages infected with mycobacteria.

For fine structure analysis, we routinely use uranyl acetate block staining, so ultra-thin sections normally carry enough contrast to be photographed with a digital camera, which sets the boundaries of the image histogram in a way that areas of highest brightness will be white while pixels of lowest brightness will be black. Thus, a high-contrast image is created, which can be evaluated without further processing. Nonetheless, for print quality images further contrasting of the section (e.g., with lead citrate) is indispensable to avoid the introduction of background noise that is created by stretching the histogram boundaries on a weakly contrasted image. Furthermore it is uncomfortable to evaluate a weakly or noncontrasted section on the TEM screen.
3. Comparison of Different Embedding Media

Polybed 812 has displaced classical Epon 812 and is now the probably most widely used resin. Completed by NMA (Nadic Methyl Anhydride) as epoxy resin, DDSA (Dodecenylsuccinic Anhydride) as hardener, and DMP-30 (2,4,6 Tris (dimethylaminomethyl)phenol as accelerator it gives hard blocks, easy to section and stable under the electron beam even under high emission current (∼35 μA). Of course, the ratio of components can be varied to alter the hardness of the blocks. Although Polybed 812 has many advantages, it has limited value for preparations of microorganisms with waxy cell walls (mycobacteria, Fig. 6A) or for high density organisms like *Staphylococcus* (Fig. 6E) or for elementary bodies of *Chlamydia* (Fig. 6M). Polybed will not penetrate the particles sufficiently; the resulting sections are instable and may even have holes. For embedding of dense material, the use of media with reduced viscosity is advisable.

Fig. 5  Comparison of light microscopy of semi-thin section and low power TEM. The images show macrophages infected with mycobacteria. The semi-thin section (A) was stained with toluidine blue and shows a lot of features visible in detail in the TEM micrograph (B). Scale bars: 5 μm.
`Spurr's` low-viscosity embedding medium was developed in the late 1960s primarily to meet the needs of botanists for a low-viscosity resin that would more easily penetrate the cell walls. It consists of four components:

```
A B C D
E F G H
I J K L
M N O P
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Fig. 6 Comparison of different embedding media. The widely used Polybed 812 will not penetrate efficiently into mycobacteria and staphylococci resulting in holey unstable sections (A, E). More reliable results are obtained with low-viscosity media like Spurr’s (B, F) and Embed-It (C, G). Due to reduced lipid extraction, cryopreparations can reveal more details (D, H). (columns from left to right: Polybed, Spurr’s, Embed-It, Cryopreparation). A–D: Mycobacteria (BCG), E–H: **Staphylococcus aureus**, I–L: **Neisseria meningitides**, M–P: **Chlamydia trachomatis**. Scale bars: 500 nm.

“Spurr’s” low-viscosity embedding medium was developed in the late 1960s primarily to meet the needs of botanists for a low-viscosity resin that would more easily penetrate the cell walls. It consists of four components: Based on the
cycloaliphatic diepoxide vinylcyclohexene oxide (VCD), it uses the epoxy resin D.E.R. 736 (diglycidyl ether of polypropylene glycol) as flexibilizer, in addition to NSA and accelerator dimethylaminoethanol (DMAE). The single components allow to specify the properties of the cured blocks depending on the respective purpose, like rapid polymerization or desired block hardness. “Spurr’s” viscosity of 60 cps leads to a better and quicker penetration of hydrophobic material, such as cell walls of Gram positive bacteria (Fig. 6F) and very dense structures such as spores, elementary bodies of Chlamydiae (Fig. 6N), and yeast cell walls. Its liquidity makes it easy to work with. Specimens sediment quickly, so less material is lost during resin exchange. Spurr resin unfortunately has less than ideal characteristics for ultrathin sectioning. It tends to stick to the cutting edge of the diamond knife and produces folded aggregates of sectioned materials. To resolve this problem antistatic devices can be used during cutting to obtain a consistent and steadily produced ribbon of sections. Another disadvantage is the instability of Spurr resin in the electron beam. It rips very quickly on exposure to the beam and is even more sensitive after lead citrate staining. To overcome this problem, the sections can be relatively stabilised by starting observations at low magnification (1000×) and low emission current (ca. 15 μA).

Embed-It™ (Polysciences, Inc.) was created as an easy-to-mix polymer of low-viscosity (65 cps) and also to avoid inconsistency between blocks. The “Spurr’s” derivative consists of two nonhazardous components, its viscosity is similar to Spurr’s, while the stability of the sections in the TEM is comparable to Polybed.

If the Polybed mixture does not penetrate samples sufficiently, it may help to prolong incubation times before changing to a different resin. A last incubation step overnight in 100% resin will produce better results, as will additional steps using different intermediate/resin ratios (3:1, 2:1, 1:1, 1:1.5, 1:2, 1:3). Changing the ratios too quickly may cause excessive shrinking and lead to holes in the specimen at, e.g., the space of a phagosome, between the phagosomal membrane and enclosed bacteria.

The comparison of the different resins shows that although all three are suitable for gram negative bacteria (e.g. Neisseria, Fig. 6I–K), Polybed is recommended because of easier section handling. Besides that, it is less toxic than VCD. Very dense structures such as elementary bodies of Chlamydiae, spores, gram positive bacteria (e.g., Staphylococcus) are better and quicker penetrated by Spurr’s resin, although even Polybed works sufficiently well in most cases. Polybed embedding is however completely unsuitable for highly hydrophobic structures such as the waxy cell walls of mycobacteria (Fig. 6A). These structures need resins like Spurr’s (Fig. 6B and F) or Embed-It (Fig. 6C and G). The latter is easier to handle than Spurr’s and is stable under the electron beam; however it cannot totally replace Spurr’s resin, since it needs longer incubation times due to its higher viscosity. Spurr’s is definitely the resin of choice for quick embeddings of dense or hydrophobic structures. It is noticeable that Spurr’s produces a slightly weaker contrast than Polybed or Embed-It sections. Contrast can be improved by longer incubation times of lead citrate but this may produce artefacts due to lead precipitation.
This is the standard embedding protocol for glutaraldehyde-fixed tissue culture specimens:

10' three washings with PBS
60' 0.5% osmium tetroxide in distilled water
10' 4 washings with distilled water
60' 0.1% tannin in 20 mM HEPES buffer
10' four washings with 1% Na₂SO₄ in 20 mM HEPES
10' four washings with distilled water
60' 2% uranyl acetate in distilled water
5' each in 30/50/70/80/90% ethanol
3 × 5' 100% ethanol
30' styrene, over night 1:2 styrene/resin
60' 1:3 styrene/resin
3 × 60' freshly prepared resin over night resin 1–2 days embedding at 60 °C
(don’t exceed 24 h for “Spurr’s”)

C. Resin Embedding of Infected Tissue

For embedding of infected tissue, it is essential to keep the size of the tissue specimen small (less than 2 mm x 2 mm x 2 mm). It is advisable to select the areas of an infected organ that will be embedded with a stereo microscope and only dissect parts that promise to be highly interesting, for the EM analysis does not allow screening of larger tissue sections. Because fixative and solvent penetration takes place over considerably larger distances in tissue, incubation times should be at least five times longer than for cell culture specimens.

It is possible to reanalyze tissue that has been embedded into paraffin for routine histology. Of course, fixation in formalin as well as heating to 65 °C are detrimental for tissue fine structure, but in some instances the reembedding into resin and subsequent EM analysis can be helpful. An advantage of this method is that particularly interesting tissue areas can be identified in sections using histological staining or light-microscopy immunodetection methods. Small cubes including these areas are then cut out of the paraffin block using a scalpel, rehydrated slowly, postfixied with glutaraldehyde and osmium tetroxide and reembedded using the standard embedding protocol. Care must be taken to remove the paraffin wax completely (Gonzalez-Angulo et al., 1978), otherwise penetration with the resin will not be successful.

After embedding, tissue blocks are transferred to cavities of flat embedding forms, laser printed identification tags are inserted and the cavity is filled with fresh resin. The sample is aligned in parallel to the tip of the cavity to ensure quick trimming and the option to section the entire tissue block surface.
D. Rapid Processing for TEM

It can be desirable to shorten the preparation of TEM specimens, e.g., of diagnostic samples. Resin embedding using microwave-assisted tissue processing reduces embedding time down to 4–5 h (Schroeder et al., 2006); alternatively cryomethods can be used which can provide better ultrastructure (compare Fig. 6I–K to Fig. 6L) since less material gets extracted compared to treatment with organic solvents (Korn and Weisman, 1966).

E. Preembedding Immunodetection Methods

Conventional resin techniques are compatible with immunodetection if the antibodies are employed before embedding. If the antigens of interest are extracellular, antibodies can be incubated with living cells on ice (to limit internalization of antibody complexes) or with cells fixed with formaldehyde (2–4% in PBS) for 30–120 min. Cells are then washed (living cells with ice-cold PBS, and then fixed with PFA), before incubation with the secondary antibody which is coupled to gold colloids (30–60 min at RT). After washing, cells are postfixed using 2.5% glutaraldehyde in PBS, and embedded conventionally. This method results in good ultrastructure combined with excellent detection of surface antigens.

Intracellular antigens are only accessible after limited permeabilization. The choice and concentration of the detergent depends on the localization of the antigen, i.e., how many membrane systems have to be crossed by the antibody before it can bind to its antigen. Our first choice is saponin at concentrations around 0.1%. Owing to the permeabilization, loss of ultrastructure cannot be avoided. To assure good penetration, antibody fragments (Fab2) should be employed, and the diameter of the gold should be less than 10 nm. Alternatively, ultra small gold (1 nm diameter or less) can be used which has to be silver-intensified before embedding (Danscher, 1981). In this case, osmium tetroxide should be omitted. Quantum Dots can be used in a similar way as ultra small gold probes. They offer the advantage that the staining can be analyzed on the light microscopic level, before the Quantum Dots are silver enhanced and processed for electron microscopy (Stoltenberg et al., 2007).

F. Immunodetection Using Ultrathin Cryosections

For more than thirty years, ultra-thin sections of frozen material have been used for immunodetection (Tokuyasu, 1973, 1978). Although in the meantime antibody labeling methods with specialized resin techniques have been developed, cryosections are still widely used since the antigen preservation in weakly fixed frozen material is superior to resin techniques. Furthermore, extraction of cytoplasmic material is reduced. The structural preservation in ultra-thin cryosections is often of poorer quality than in resin sections mainly due to the softer fixation methods employed.
Structural preservation and antigen reactivity have to be balanced to obtain optimal results. The contrast in TEM images of cryosections is weaker and less differentiated than in osmicated samples of resin sections. Although fine-structure resolution in resin sections is often superior, EM-sample preparation by cryosectioning takes less time. Also, problems with resin polymerization in bacteria that show a particularly resistant cell wall will not come up under cryosectioning conditions.

Samples of infected cells for cryosectioning can be prepared from adherent cells grown in 6-well culture plates. One sample should consist of at least one confluent well, or preferably two wells (>10⁶ cells). It is important to achieve a high infection rate in order to obtain enough representative cross sections of pathogens in the resulting ultra-thin sections. Cell density and infection can be monitored with an inverted light microscope using phase contrast illumination.

Fixation of the infected cells should take place at incubation temperature to prevent artefacts due to temperature shock. Fixation in PBS or in the growth medium works well in most cases. In some cases further improvement in structural preservation is observed when fixing the cells in cytoskeleton stabilizing buffer (1 mM EGTA, 4% polyethylene glycol 6000 (8000), 100 mM PIPES pH 6.9, (Lindroth et al., 1992)). A combination of 2–4% PFA with 0.05% glutaraldehyde, applied for two hours is a good starting point for many antigens and results in acceptable structure preservation combined with sufficient antigen reactivity. This is discussed in detail in the classic book by Griffith (1993).

Infected cells are harvested with a rubber scraper and suspended in 10% gelatine in PBS at 37 °C and immediately centrifuged to sediment as a pellet. This is excised from the tube after cooling and gelatinizing and cut into smaller pieces fit for infiltration in sucrose solution (2.3 M sucrose in sodium phosphate buffer with 1% PFA, pH 7.4) After infiltrating preferably in a cold room and on a slow inverting shaker overnight the gelatin bits can be mounted on aluminium stubs and frozen in liquid nitrogen for cryosectioning. Sectioning is carried out at −120 °C. Sections are transferred to carbon-coated pioloform films on copper or nickel EM-grids and kept section side down on 2% gelatine until immunolabeling and contrasting. For a detailed description of cryosectioning techniques see Chapter 8, this volume.

A basic immunogold labeling of ultra-thin cryosections is carried out as follows: After blocking the sections by floating the grids on drops of blocking buffer (1% BSA, 0.02 M glycine, 10% cold water fish gelatine in PBS) for at least 30 min. Primary antibodies are applied at 1–10 μg/ml diluted in blocking buffer. After incubating for 1 h at 37 °C or over night at 4 °C in a humid chamber, excess antibody is removed by washing 6 × 3 min on drops of PBS. Secondary antibody coated gold colloids are applied for 30 min–1 h at 37 °C diluted in blocking buffer. The labeled grids are washed 6 × in PBS and 5 × in distilled water prior to negative contrasting and embedding in methyl cellulose. For this, the sections are floated on three consecutive drops of 0.2% uranyl acetate and 2% methyl cellulose in distilled water for 2.5 min each before blotting off excess contrasting solution and air drying the grids.
Again, this technique can be correlated with light microscopy. Ribbons of semithin sections can be transferred to glass coverslips together with the pick-up solution. After washing the coverslip on three consecutive drops of PBS, the sections can be stained with a DNA dye and mounted for quick viewing with a fluorescence microscope or used for immunofluorescence staining. The comparison between immunogold labeling and immunofluorescence can help to secure staining specificity if the antigen is only present in small amounts. While intense immunogold staining that correlates with morphology (Fig. 7) is easy to interpret, specificity of a weak staining especially of cytoplasmic antigens can more reliably assessed if correlated with immunofluorescence.

G. Scanning Electron Microscopy

1. Scanning Electron Microscopy of Cultured Cells

For Scanning Electron Microscopy (SEM) analysis, we routinely grow cells on round coverslips (dia. 13 mm). Cells are fixed by adding the fixative stock solution to the medium at 37 °C. If no immunodetection is intended, we use glutaraldehyde at a final concentration of 2.5% for 120 min at room temperature. The fixative is directly added to the cell cultures to avoid loss of material or changes in the pathogen/host interplay. Thus, even fragile structures like NETs (see Fig. 8) can be sufficiently stabilized to survive the consecutive preparation steps.

After several washes with H₂O, the specimens are postfixed for 30 min with OsO₄ to stabilize membranes. Cells are washed repeatedly with H₂O, and then transferred for 30 min to a buffered solution of tannic acid (0.5% in 20 mM HEPES). Repeated treatment with osmium and tannic acid will deposit a

Fig. 7 Detection of mycobacterial antigens on ultrathin cryosections of infected macrophages. The indirect immunogold method using 12 nm gold colloids reveals that mycobacterial antigens are exclusively located inside the phagosome. Scale bar: 500 nm.
conductive layer on the cells which helps minimizing surface charging to a degree that no additional metal layer will be necessary if the specimens are to be analyzed at low acceleration voltage (<1 kV). After the last treatment with osmium, cells are dehydrated using a graded ethanol series. We use molecular sieves to keep the 100%-ethanol water free which requires the filtration of the dry ethanol to remove debris of the molecular sieves. After three dehydration steps using 100% ethanol, cells are transferred to a critical point drying apparatus equipped with a holder for the coverslips, and dried. Since we regularly examine specimens at 20 kV, we coat the surface of the dry cells with 3–5 nm of platinum/carbon. This results in a coating with a finer grain than sputtering them with gold or gold–palladium.

2. Immunodetection Using SEM

For detection of surface antigens, we fix the cells by adding stock solutions of PFA to the warm medium to a final concentration of 4%. After 30 min at room temperature, the specimens are washed with PBS and blocked using 1% BSA in PBS. Primary antibodies are diluted in the same buffer at a concentration between 1 and 10 μg. The coverslips with the cells facing the bottom is placed on 50 μl drops of the antibody solution on parafilm in a humid chamber and incubated at 37 °C for 30–60 min. After repeated washing, the secondary antibody coupled to gold colloids is applied correspondingly. Although the labeling density is lower, we prefer medium-sized gold particles (12–15 nm), because they are more readily identified in the SEM than smaller particles (6 nm or less).

Fig. 8  Scanning electron microscopic image of *Shigella*—infected human neutrophil granulocytes with neutrophil extracellular traps (NETs). By adding fixative directly into the culture without exchanging media, even fragile structures like NETs as well as bacteria only lightly adhering to cells surfaces can be retained. Scale bar: 5 μm.
We take material contrast views with the backscattered electron detector (BSE) (Fig. 9A) and topographic images with the secondary electron (SE) detector (Fig. 9B). Even when the specimens are coated with a layer of 2–3 nm platinum/carbon, the gold signal is clearly visible with the BSE detector. Using individual images from both type of detectors normally allows easier identification of gold signal than images created with mixed signal from both detectors at the same time. For presenting both images in an overlay, SE and BSE micrographs can be pseudocolorized using different look-up tables, (e.g., green and red) (Brinkmann et al., 2004).

III. Overview and Conclusion

In this article we have presented some standard preparations for both TEM and SEM, which will be helpful to infection biologists who would like to get a more precise view on the process of infection. Yet certain caveats have to be kept in mind. Compared with that in light microscopy, in electron microscopy the number of individual cells in a given sample is normally much smaller. Great care has to be taken to assure that an adequate number of individual cells are analyzed to represent the entire sample; thus, it is of great importance to elaborate infection
parameters that ensure homogenous infection patterns in cell culture or organ samples. It is a good idea to correlate light and electron microscopy to be able to monitor the infection status of the entire sample before concentrating on fine structure analysis of a relatively small number of cells.

If knock-down techniques are used to repress expression of a certain gene, it is necessary to carefully monitor the degree of gene silencing. While primary cells or organs from mice with properly induced KO germline mutations will surely not express the protein under investigation, silencing genes with RNA techniques never repress protein expression entirely. Often, 20–40% of residual expression remains, which may be enough to yield a readout using light microscopic techniques, but is not satisfactory for electron microscopy, which concentrates on a limited number of individual cells. Thus, for EM studies RNA-induced gene silencing is only adequate if a high repression rate (>90%) is accomplished, and the number of analyzed cells is big enough to be statistically significant.

For a researcher new to transmission electron microscopy it is often dissatisfactory that the number of infected cells in a given sample seems to be much smaller than in a corresponding sample processed for light microscopy. It has to be kept in mind that while light microscopy always displays the entire cell and even a single bacterium infecting this cell can easily be visualized, an ultra-thin section of 60 nm only represents about 0.5–1% of the cell, and thus many cell profiles erroneously appear uninfected. This again is an argument for correlative studies.

The multiple facets of electron microscopy offer indispensable tools for studying infection processes. We would like to encourage more biologists to use electron microscopy in their infection models to gain a more precise insight into the biology of infection.

References


The future is cold: cryo-preparation methods for transmission electron microscopy of cells

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Our knowledge of the organization of the cell is linked, to a great extent, to light and electron microscopy. Choosing either photons or electrons for imaging has many consequences on the image obtained, as well as on the experiment required in order to generate the image. One apparent effect on the experimental side is in the sample preparation, which can be quite elaborate for electron microscopy. In recent years, rapid freezing, cryo-preparation and cryo-electron microscopy have been more widely used because they introduce fewer artefacts during preparation when compared with chemical fixation and room temperature processing. In addition, cryo-electron microscopy allows the visualization of the hydrated specimens. In the present review, we give an introduction to the rapid freezing of biological samples and describe the preparation steps. We focus on bulk samples that are too big to be directly viewed under the electron microscope. Furthermore, we discuss the advantages and limitations of freeze substitution and cryo-electron microscopy of vitreous sections and compare their application to the study of bacteria and mammalian cells and to tomography.

Introduction

Since the invention of the TEM (transmission electron microscope), the basic principle of TEM remained unchanged: the TEM column is under vacuum, allowing a beam of coherent electrons to be directed on to the sample. Due to the scattering of electrons within the sample, only small objects can be directly viewed. Larger samples need to be sectioned for their analysis. As a consequence of their water content, most biological samples are too soft to be sectioned thinly enough without preparation. Most importantly, in the vacuum of a TEM column at room temperature, water of an unprepared sample would evaporate, damaging both the sample and the TEM.

Therefore many preparations of biological samples for EM (electron microscopy) remove the water. In the simplest case, as in negative staining, the dehydration of the sample is by air-drying. Bigger samples are first chemically fixed before the water is replaced by an organic solvent that allows the infiltration with a plastic resin. Once the resin is cured, the formerly soft biological samples are hard enough to be cut into thin sections that can be viewed under a TEM. In this preparation the chemical fixation has two goals: it arrests the biological activity of the sample and stabilizes it for further processing. Alternatively, rapid freezing can arrest the biological processes of a sample, as long as it remains frozen.

In the present review, we give a short introduction to the sample preparation that uses chemical fixation to arrest the biological activity. We compare this with methods of rapid freezing for bulk samples and the subsequent processing for TEM: dehydration by FS (freeze substitution) or CEMOVIS (cryo-electron microscopy of vitreous section). A list

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Key words: cryo-electron microscopy of vitreous section (CEMOVIS), freeze substitution, high-pressure freezing, tomography, transmission electron microscopy.

Abbreviations used: CCD camera, charge-coupled-device camera; CEMOVIS, cryo-electron microscopy of vitreous sections; CETOVIS, cryo-electron tomography of vitreous sections; CM, cellular membrane; EM, electron microscopy; ET, electron tomography; FS, freeze substitution; HPF, high-pressure freezing; LPS, lipopolysaccharide; OM, outer membrane; SNR, signal-to-noise ratio; TEM, transmission electron microscope/microscopy.
of handbooks for further reading that give practical insights is presented in Table 1.

### Conventional preparation for TEM

We use this term for all preparation procedures that use chemical fixation and the following preparation steps at room temperature.

#### Chemical fixation at room temperature

Most of the time aldehydes are used for chemical fixation. They are dissolved in a buffer and mainly react with amino groups in proteins and amino lipids. Aldehydes thereby cross-link proteins and certain lipids and immobilize them. However, the fixative starts to react as soon as it comes in contact with the specimen. This leads to a discontinuity in time of fixation; fixatives rely on diffusion to penetrate the sample and are consumed by reaction with the sample (Griffiths, 1993). Thus a gradient is formed during penetration of the fixative into the volume of the sample, which inactivates first the outermost region, whereas the interior remains unfixed. Since EM preparation consists of several steps, it is sometimes not easy to assign an artefact to one part of the procedure. However, the formation of membrane blebs in the case of sea-urchin eggs has been demonstrated as a consequence of chemical fixation (Chandler, 1984). The reaction of aldehydes with lipids is limited and, even if the lipids are still in place after aldehyde fixation, they might be extracted in the following steps. To stabilize lipids, osmium tetroxide (OsO$_4$) is used, which reacts mainly with unsaturated lipids but also with amino acids. Prolonged incubation at room temperature with OsO$_4$ can lead to the cleavage of proteins and alterations in their structure (Baschong et al., 1984).

#### Dehydration and embedding in a plastic resin

To achieve sufficient stability for thin sectioning, the sample is embedded in a plastic resin, either an epoxy or a methacrylic resin. Most resins are hydrophobic and do not mix with water. The sample needs first to be dehydrated to enable infiltration of the resin. Thereby the water of the sample is replaced gradually by an organic solvent, mostly ethanol or acetone. Biochemists and molecular biologists use both reagents at room temperature to precipitate proteins or DNA

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**Table 1: Handbooks for further reading**

<table>
<thead>
<tr>
<th>Book title</th>
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<tr>
<td>Methods in Cell Biology, volume 79 (Cellular Electron Microscopy)</td>
<td>Covers a wide spectrum of technical approaches such as EFTEM, EFSTEM, tomography and tomography.</td>
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because they are not soluble in organic solvents and form aggregates. The chemical fixation prior to dehydration prevents major deteriorating effects of the solvent because cross-linked proteins are insensitive to aggregation. Still, not all compounds of the sample are immobilized by chemical fixation: bacterial DNA contains a small amount of DNA-binding protein compared with mammalian cells. It is therefore not cross-linked by conventional chemical fixation and forms aggregates during dehydration (Hobot et al., 1985). Also lipids can retain their mobility after chemical fixation and are extracted not only by the solvent but also by the plastic resin during infiltration (Paul and Beveridge, 1992). After infiltration of the sample the resin is polymerized either by heat, as for epoxy resin, or by UV light for methacrylic resin.

Hardening the sample without resin: the Tokuyasu technique

This technique is mainly used for immunohistochemistry studies. It avoids dehydration and infiltration with a resin. Instead the sample is frozen to harden it for thin sectioning. The first experimental approach of this was done by Fernández-Morán (1952). Fernández-Morán’s attempts and subsequent efforts were limited by freeze damage. It was a breakthrough when cryo-protectants were introduced to avoid freeze damage (Tokuyasu, 1973). As in the case of conventional preparation the sample is cross-linked by chemical fixation using aldehydes. Afterwards it is infiltrated with a high-molarity sucrose solution as a cryo-protectant, which is possible because chemical fixation permeabilizes the CM (cellular membrane) for small molecules (Penttilla et al., 1974). The sucrose avoids ice crystal formation, and samples up to a volume of 1 mm³ can be vitrified by plunging in liquid nitrogen (Griffiths et al., 1984).

From the frozen sample, thin cryo-sections are prepared and picked up with a loop containing a small volume of sucrose solution or a mixture of methylcellulose and sucrose (Liou et al., 1996). The sections are then thawed at room temperature and transferred on to a grid that has previously been coated with a Formvar film and a carbon layer. The sections can be labelled with any affinity probe, mostly antibodies, lectins, but also in situ hybridization for mRNA localization is possible (Slot and Geuze, 2007; Herpers et al., 2010). The labelling is visualized by a probe linked to colloidal gold (Slot and Geuze, 1981). After the labelling procedure, the section is embedded in a mixture of uranyl acetate and methylcellulose (Griffiths et al., 1982). This step is crucial because it provides contrast and dries the sections. The collapse of subcellular structures is prevented by the layer of methylcellulose, which protects the sample from surface tension during drying (Tokuyasu, 1978). This technique yields an excellent visualization of membranes. Together with the localization of antigens, this has resulted in major contributions in the field of intracellular transport in mammalian cells. However, it also illustrates how affinity to the heavy metal stain determines the image one obtains. The cytoplasm of a mammalian cell in a conventional preparation is full of ribosomes, which are difficult to visualize with the staining of Tokuyasu sections.

Rapid freezing as an alternative to sample immobilization

In the Tokuyasu technique, freezing is utilized to avoid resin embedding, but freezing can also be used to arrest cellular processes simultaneously and independent of their reactivity with a chemical fixative. Different methods of freezing and subsequent processing exist (Figure 1). Before going into the details of these methods it is first necessary to look at the characteristics of the main component of the sample: water. Comprehensive overviews covering the properties of water and its freezing have been given by Dubochet (2007, 2009).

Freezing of water

The water molecule is a dipole with a positive charge of the two hydrogen atoms and a negative charge of the oxygen atom (Figure 2A). Water molecules can interact with each other via hydrogen bonds. In total, one water molecule can form four hydrogen bonds and is then the centre of a tetrahedron (Figure 2B). The absence of these hydrogen bonds characterizes the vaporous state of water. In contrast, when water is present as ice, the molecules are in a crystalline array,
Figure 1 | Schematic overview of different cryo-preparation methods

The diagram shows the most commonly used methods for rapid freezing and the subsequent sample preparation. The first column on the left shows the generalized steps with preparation steps or observation in italics: the starting point is the living sample, which is rapidly frozen. The vitrified sample is next prepared for the observation in the microscope. In the upper row the samples are divided according to their size. The size determines the method of rapid freezing. After freezing, the water of the sample is vitrified and immobilizes the sample (middle row of boxes). From this point, various routes for sample preparation are possible that lead to different types of imaging (lower row of boxes). RT, room temperature.

where each molecule forms four hydrogen bonds with other water molecules (Figure 2C). The molecular organization of the liquid state of water is probably in between that of the vaporous and ice states. Water molecules, in a liquid, form hydrogen bonds but the molecules are mobile because hydrogen bonds can be bent and broken.

Freezing is the phase transition from liquid water into ice. Freezing requires a nucleation event which gives the seed for ice crystal growth (Dubochet et al., 1988). The cooling rate determines the growth rate of the crystal as well as the form of ice. At ambient pressure slow cooling results in the formation of hexagonal ice, whereas, with faster cooling, cubic ice is formed. At higher cooling rates, water molecules are vitrified and immobilized before a nucleation event of an ice crystal can take place (Brüggeller and Mayer, 1980; Dubochet and MacDowell, 1981). The vitrified water can be considered as a simplified definition of a liquid, which has a very high viscosity.

Freezing of biological samples

What does this imply for the freezing of biological samples? Despite the water content, a biological material is different from pure water. Cells contain soluble material such as ions, proteins and sugars that interact with the water molecules and thereby change their freezing behaviour. Solutes decrease the temperature for nucleation events and increase the temperature at which water becomes vitreous (MacKenzie, 1975). The aim of rapid freezing is to immobilize the sample without a change in the morphology. Therefore ice crystal formation has to be avoided. A growing ice crystal will exclude all the solutes from the
Cryo-preparation of cells for transmission electron microscopy

Review

Figure 2 | From the water molecule to ice
A schematic view of the water molecule is shown. (A) A single water molecule. The oxygen atom (red) attracts stronger electrons of the covalent bonds with two hydrogen atoms (white). This results in a positive charge ($\delta^+$) at the hydrogen atoms and a negative charge ($\delta^-$) at the oxygen. (B) The tetrahedron has a water molecule in the centre, which forms hydrogen bonds with four other water molecules. (C) The smallest unit of hexagonal ice.

crystal (Dubochet et al., 1988). As a result the cell is separated into ice crystal(s) and concentrated solutes. Therefore the water in the cell should always be vitrified and thus it immobilizes all the solutes.

How to achieve vitrified samples
To vitrify the water in a sample, high cooling rates are required, which are applied to the surface of the sample. The thermal conductivity of water is poor and the cooling rates decrease with the depth of the sample, which favours ice formation. Therefore, the choice between different freezing techniques depends not only on the microscopy approach but also on the size of the sample.

Plunge freezing
The immersion of small biological objects within a thin film of water into a cryogen has become a widely used method, in which the sample is embedded in a thin layer of vitrified water and is subsequently directly observed under a cryo-microscope (De Carlo, 2009). It can be applied to isolated protein complexes, viruses, bacteria and single cells. For bulk samples such as larger cells and tissue, this approach is not suitable, as the sample is too thick to be observed directly under the microscope, and it is probable that not the entire sample has been vitrified.

Slam freezing
Larger samples can be frozen by slam freezing (Escaig, 1982). In this approach the sample is propelled on to a polished metal surface, which is cooled by liquid nitrogen or helium. The surface of the sample is in direct contact with the cooled metal plate and receives high cooling rates. From the surface of the sample the poor thermal conductivity of the biological material limits the extraction of heat and a temperature gradient builds from the surface to the interior. The depth of vitrification achieved by this method depends on the water content of the sample, but it is generally considered that up to 10–20 $\mu$m samples can be frozen without visible ice crystal formation (Escaig, 1982).

HPF (high-pressure freezing)
To freeze larger samples the freezing behaviour of water needs to be modified. One possibility to achieve this is the use of a cryo-protectant as in the Tokuyasu technique. Another possibility, in a non-invasive way, is by HPF (Riehle and Hoechli, 1973). This method is based on the reasoning that nucleation of ice crystals is both temperature- and pressure-dependent. The transformation of water from the liquid to the crystalline state is accompanied by an increase in volume, which is hindered by the application of high pressure. Under 2100 bar (1 bar = 100 kPa), the melting point of water as well as the temperature for the nucleation of an ice crystal in pure water are lowered to $-22$ and $-92\,^\circ C$ respectively (Kanno et al., 1975). As a consequence lower cooling rates are required to vitrify samples compared with freezing at ambient pressure, and samples can be vitrified up to a thickness of 200 $\mu$m (Riehle and Hoechli, 1973; Studer et al., 1995). However, vitrification of pure water or dilute aqueous solutions by HPF is not possible (Sartori et al., 1993). Luckily, most compounds of the cell act as a natural cryo-protectant and vitrification can be achieved (Studer et al., 1995). To avoid

Vitrification: Transformation of a material into an amorphous, ‘glass’ state.
ice crystal formation outside the cell, an extracellular cryo-protectant can be added (McDonald, 2007).

Before freezing, the high-pressure freezer applies for a few milliseconds a pressure of 2100 bar to the sample, which raises the question of whether the pressure has an impact on the sample. However, it was shown that the viability of the green alga *Euglena gracilis* was only mildly affected by the short time the organisms were subjected to high pressure (Riehle and Hoechli, 1973). In addition, the cell wall of *Mycobacterium bovis* and *Mycobacterium smegmatis* was analysed after plunge freezing and cryo-electron tomography as well as after HPF and cryo-sectioning. Both methods delivered the same information on organization and measured membrane thickness (Hoffmann et al., 2008). Still it should be noted that HPF does not preserve the cholesteric structure of DNA and induces changes in the structure of liposomes consisting of two different phospholipids (Leforestier et al., 1996; Semmler et al., 1998).

**Handling the water after freezing**

After freezing, the sample is immobilized as long as the water remains vitrified. Further processing of the sample depends on its size.

Small samples that were frozen by plunge freezing can be directly observed under a cryo-microscope. The grid is observed in a cold stage holder at close to $-180^\circ$C. At this temperature the evaporation rate of water becomes insignificant and it is possible to observe the hydrated sample in the vacuum of the microscope (Dubochet et al., 1982). A comparison of macromolecular assemblies analysed by negative staining or by embedding in vitreous water and cryo-EM is given elsewhere (Hoenger and Aebi, 1996).

Larger samples need to be sectioned first. For sectioning the samples, either of two procedures can be chosen (Figure 1): (i) dehydrate the sample at low temperature and embed it in a plastic resin, a procedure called FS, or (ii) keep the sample frozen and hydrated and then cut it into thin sections, which can be observed under the cryo-microscope (CEMOVIS).

**Freeze substitution**

Although FS consists of dehydration and often uses chemical fixation, it differs from the above-described conventional preparation in the order of the events. During FS the sample is first dehydrated at low temperatures before it is chemically fixed (Steinbrecht and Müller, 1987).

**Dehydration in the cold**

The water of the sample is dissolved at $-90^\circ$C by an organic solvent such as methanol or acetone. Whereas pure, vitrified water transforms into cubic ice at the devitrification temperature $T_v \approx -135^\circ$C, this value is expected to be higher for biological material because of the cellular components. But it is currently not known whether water is in the vitreous state or has transformed into cubic ice during substitution (Dubochet, 2007).

In cases where chemical fixatives are added to the solvent, the fixatives commonly used are not reactive at $-90^\circ$C: therefore the introduction of FS was, at first, critically received. It was questioned whether the dehydration of the sample before the sample is chemically fixed allowed extraction of lipids or promoted the aggregation of proteins. However, the interactions between solvent and biological material are temperature-dependent. In addition, the viscosity of the solvent increases with decreasing temperature and stabilizes the sample, because it limits the mobility of proteins and their capability to form aggregates (Kellenberger, 1991). This model is supported by the use of solvents alone without chemical fixation as long as embedding of the sample and polymerization of the resin are performed at low temperatures (Weibull et al., 1984; Humbel and Müller 1985).

**Chemical fixation and embedding**

When samples are embedded and polymerized at room temperature, addition of chemical fixatives during substitution is obligatory. Usually, OsO$_4$, uranyl acetate and aldehydes are added separately or in combination, and a variety of protocols can be found in the literature. More recently, epoxy resin was introduced as a fixative during FS (Matsko and Müller, 2005). The activity of these fixatives is temperature-dependent: OsO$_4$ starts to react at $-70^\circ$C, uranyl acetate is supposed to react at even lower temperatures and glutaraldehyde begins to cross-link macromolecules at $-50^\circ$C (White et al., 1976; Humbel and Müller, 1985). Even though the fixative is inactive during substitution at $-90^\circ$C, it penetrates the sample and can react simultaneously as soon as the temperature is raised. In this way the gradient of fixative during...
Cryo-preparation of cells for transmission electron microscopy

Review

conventional preparation is avoided. The importance of this for morphological preservation is highlighted by the formation of mesosomes in bacteria. This membranous compartment forms when bacteria are fixed at room temperature with osmium, whereas it is absent when osmium is used at low temperatures during FS (Ebersold et al., 1981).

Compared with conventional preparation, HPS/FS samples often have less contrast, especially for membranes (Walther and Ziegler, 2002). One possible explanation for this phenomenon is the good preservation of proteins surrounding the membranes that inhibit access of the stain (Matsko and Müller, 2005). A number of methods for enhancing membrane contrast during FS have been described: addition of small amounts of water to the substitution medium (Walther and Ziegler, 2002), the substitution medium containing tannic acid in combination with OsO₄ (Jiménez et al., 2009), or glutaraldehyde and uranyl acetate in acetone (Giddings, 2003).

Freeze-substituted samples are embedded in either epoxy or methacrylic resins. The former provides good structural preservation and is more stable under the electron beam, and the latter may be better suited for immunocytochemical experiments, since impregnation and UV polymerization can be performed at low temperatures (Schwarz and Humbel, 1989; Monaghan et al., 1998). In addition, there is less interaction between proteins and methacrylic resins compared with epoxy resin, which reacts with proteins (Kellenberger et al., 1987). During the last few years, protocols were also developed to adapt HPF/FS for subsequent immunolabelling according to the Tokuyasu technique (van Donselaar et al., 2007; Ripper et al., 2008).

CEMOVIS

The basic principle of CEMOVIS is straightforward. (i) The sample is frozen to vitrify the cellular water. (ii) The vitrified sample is sectioned in a cryo-microtome below \( T_v \) and the sections are transferred to an EM grid. (iii) While keeping the sections on the grid below \( T_v \), the grid is transferred to a cryo-TEM, where it is observed at close to \(-180^\circ C\) under low-dose conditions (McDowall et al., 1983)

As the direct observation of small samples embedded in vitreous water, CEMOVIS avoids chemical preparation steps (chemical fixation, dehydration and staining with heavy metals) and the sample is observed as close to the native state as possible. In addition, only cryo-EM allows the unequivocal proof of vitrification when the state of the water is analysed by electron diffraction (Dubochet et al., 1982).

Freezing of CEMOVIS samples

Because HPF increases the depth of vitrification, it is often the method of choice for CEMOVIS. While cellular contents act as internal cryo-protectants, vitrification of the medium around the cells is achieved by addition of a cryo-protectant. Vitrification of cells and the surrounding medium is important, because hexagonal or cubic ices make the sample brittle for sectioning. Most commonly, a 20% (w/v) solution of a high-molecular-mass dextran (40 kDa) is used for freezing samples, which has a low osmotic value (20 mosm) (Dubochet et al., 2009).

Sectioning and difficulties associated with it

After vitrification the sample needs to be prepared for sectioning. The sectioning itself differs from the room temperature procedure. The knife for plastic sectioning at room temperature consists of a diamond knife with a water trough at the knife edge. Until now no fluid has been found that remains liquid at the temperatures required for CEMOVIS sectioning and has a high enough surface tension for the sections to float (Ladinsky, 2010). Therefore a dry knife is used for cryo-sectioning and the absence of a liquid to float the sections increases the interaction between the forming section and the knife (Griffiths et al., 1984). This results not only in a more demanding sectioning procedure but also in the formation of more cutting artefacts (Al-Amoudi et al., 2005).

The most obvious artefacts on a CEMOVIS image are knife marks. They are a result of an uneven knife edge and are present at the surfaces of the section (Al-Amoudi et al., 2005). Knife marks are also present on plastic sections, but normally they are not visible due to the heavy metal staining of the section and can be visualized by surface shadowing (Griffiths, 1993).

During sectioning the knife edge applies a force to the forming section. If this force is above a certain threshold, crevasses form. Crevasses are fractures on the surface of the section. Their formation is related to the thickness of the sections; while they are absent from thin sections, they are more pronounced.
when the thickness of the section increases or when the sample is not properly vitrified (Al-Amoudi et al., 2005; Sartori et al., 1996).

In addition, the force applied to the forming section results in compression, which represents a deformation of the section in the cutting direction. Compared with the blockface the length of the section is reduced, which is compensated for by an increase in section thickness. Compression depends on the angle of the knife and on the thickness of the sections: it is more pronounced when very thin sections are cut (Jésior, 1989, Han et al., 2008). The main problem of compression is that it is not homogeneous. Within the same cell, cross-sections of microtubules can show different levels of compression and it has been reported that compression differs at the cellular and the molecular level (Zuber et al., 2005; Pierson et al., 2011). With homogeneous compression the image could be corrected by the application of a mathematical model (Al-Amoudi et al., 2005), but, if different levels of compression exist within the sample, such modelling is not possible. These examples underline the need to better understand the process of cryosectioning.

Regarding the section thickness, crevasses and compression have an inverse relationship: crevasses are absent from thin sections cut with a nominal feed of 40 nm, but compression is pronounced, whereas on thicker sections (100 nm nominal feed) compression is less severe but crevasses are abundant. It is important to find the optimal conditions under which the impact of both artefacts is minimized (Han et al., 2008).

Having established the best conditions, one obtains a ribbon of sections on the knife that need to be transferred to a grid coated with a carbon film. The transfer and then the adhesion of the sections to the grid is probably the most demanding step in the whole procedure and different approaches have been developed (Dubochet et al., 2009; Ladinsky, 2010; Pierson et al., 2010).

Imaging of CEMOVIS sections

The absence of stain allows visualization of the hydrated sample in the near to native state because the image represents the real mass distribution rather than its affinity to the stain. However, when CEMOVIS images are viewed for the first time, the impression is often that this is the greyest EM image seen so far. The contrast is low compared with the heavy-metal-stained EM images. With heavy metal stain the main mode of contrast formation is amplitude contrast, where electrons can be considered as particles. Contrast is formed when the section is observed in focus with a small objective aperture. Parts of the sample with high affinity for the stain will lead to scattering of electrons. Most of the scattered electrons are removed by the objective aperture, which results in sharp contrast between areas with and without stain. In contrast, the atoms in a non-stained biological sample have similar values for their scattering amplitude and therefore will result in hardly any amplitude contrast. The main mode of contrast formation in this situation is phase contrast, in which electrons behave as a wave. The sensitivity of phase contrast reveals local differences in density. However, the information in the picture depends on the defocus: at a given defocus value certain features are amplified, whereas others are not visualized (Dubochet et al., 1988). A single CEMOVIS picture can give a high-resolution image of certain structures in the sample. It requires acquisition of images with different defocus values to collect high-resolution details from structures of different size. An example correcting for this effect is the study of mitotic chromatin organization (Eltsov et al., 2008).

Another difference between CEMOVIS sections and plastic sections is the sensitivity of the former to the electron beam. As mentioned above, the vitrified water in the sample can be considered as a liquid with very high viscosity and can be rearranged by the energy of the electrons. This is easily seen with the disappearance of crevasses and knife marks after the exposition of a certain electron dose. Both artefacts represent a three-dimensional relief on the surface of the section. The electron beam increases the flow of the section, and as a consequence of the high surface tension of water, the artefacts are levelled out (Sartori Blanc et al., 1998).

An excess of electrons also leads to ‘bubbling’: a high dose of electrons induces the formation of small gaseous fragments, which accumulate as bubbles.
within the vitrified specimen (Dubochet et al., 1982). But these artefacts can be prevented when the sections are observed using proper low-dose conditions.

The adherence of the section to the carbon film on the grid is important to obtain good images. The part of the section adherent to the carbon film is more stable during imaging (Sartori Blanc et al., 1998). Often only a part of a CEMOVIS section adheres well to the carbon film. Whether this is due to local differences in the sample or within the carbon film is not known. Therefore it will be important to gain more insights into the electrostatic charges that support or inhibit attachment of a CEMOVIS section to the carbon film, as well as into the optimal support film for the adhesion of the section.

**What does the image show?**

CEMOVIS allows one to visualize sections of bulk samples in the hydrated state. Compared with conventional preparation or FS, keeping the sample in the hydrated state comes at the price of more demanding technical steps, mainly sectioning and imaging. This raises the question of what does one gain by using a more cumbersome approach. In the following paragraphs we want to discuss this for bacteria and mammalian cells.

**Imaging bacteria: *Shigella flexneri***

Figure 3 shows a comparison of *S. flexneri* that was immobilized by HPF and either processed for FS and epon embedding or prepared for CEMOVIS. The overview shows for both techniques a remarkable dense cytoplasm of the bacteria compared with conventional preparation, which leads to aggregation of the bacterial DNA and extraction of soluble contents (Hobot et al., 1985). The cytoplasm of the bacteria is characterized by macromolecular complexes, most likely ribosomes and areas devoid of them. Compared with the high contrast of the FS image, the CEMOVIS picture is more uniform. On a first view, even the material around the bacterium has a grey level similar to that of the bacterium itself.

However, looking at the cell wall of the bacterium provides more details. In the FS preparation the OM (outer membrane) is clearly visible as a straight, electron-dense line. On its outside, a fuzzy electron-dense layer characterizes the side chains of the LPS (lipopolysaccharide) present in the outer layer of the OM. The cytoplasm of the bacterium is separated by the CM, which appears as an electron-lucent line and shows that OM and CM have different affinities to the heavy metal stain. The periplasmic space between both membranes often appears empty with conventional preparations (Hobot et al., 1984). With the FS preparation the periplasmic space is filled with a periplasmic gel, which is separated by an electron-dense layer into an outer and an inner region (Hobot et al., 1984).

In the CEMOVIS image OM and CM are clearly visible. As reported by others, the side chains of the LPS on the outer layer of the OM are not detectable (Matias et al., 2003). This is most likely because in CEMOVIS the natural mass distribution is visualized by phase contrast. The difference between the mass of the LPS side chains compared with the surrounding dextran solution is too subtle to form a contrast.

The view of the periplasmic space demonstrates the increase in resolution obtained with CEMOVIS: in the FS image, the outer periplasmic gel appeared with a uniform density, yet in the CEMOVIS picture this region is organized with a dotted structure present underneath the OM.

Using CEMOVIS for recent studies of the cytoskeleton in bacteria, the organization of bacterial DNA or the cell wall of *Mycobacterium* spp. revealed new views on bacterial morphology and highlight the necessity for close to native state imaging (Eltsov and Dubochet, 2005; Salje et al., 2009; Zuber et al., 2008; Bleck et al., 2010).

**Imaging mammalian cells: melanocytes**

Melanocytes are specialized cells mediating pigmentation of the skin. Pigmentation requires melanin synthesis and storage, which is confined to lysosome-related organelles named melanosomes. Melanosome biogenesis itself is linked to morphological and biochemical changes: mainly fibril formation in the lumen of the melanosome and the subsequent melanin deposition (Hurbain et al., 2008, and references cited therein).

Compared with conventional preparation (results not shown) the limiting membrane of the stage III melanosome is less ondulated with HPF/FS (Figure 4A). The fibrils in the lumen are partially covered by melanin. The CEMOVIS image shows as well a stage III melanosome (Figure 4B). The lower inset is a detail of the melanosome with one part of a fibril
Figure 3 | Two views of *S. flexneri*

Bacteria were frozen by HPF, followed by FS and epon embedding (A, C) or processed for CEMOVIS (B, D) as described previously (Couture-Tosi et al., 2010). (A, B) Overview of a longitudinal section of the bacterium. (B) The arrow is aligned to a knife mark and the asterisk marks an area with crevasses. (C) Detail of the cell wall. The OM (black arrowhead) shows a thin hair-like electron-dense outer layer, which represents the sugar chains of the LPS. The periplasmic space is between the OM and the CM (white arrowhead) and is separated into an inner and an outer part, separated by an electron-dense line (white arrow). (D) Cell wall on a CEMOVIS section. OM and CM are clearly visible. In the outer part of the periplasmic space a dotted structure is present underneath the OM. Scale bars: 200 nm (A, B) and 100 nm (C, D).

in the right orientation to show that the surface of the fibrils consists of a saw-like structure (white arrowhead in Figure 4(B), lower inset). In addition, the bi-layer of the limiting membrane is resolved (black arrowhead in Figure 4(B), lower inset). However, the limiting membrane of the melanosome is not visible all around the organelle. Whether a structure can be detected by phase contrast depends on the orientation and dimension of the structure within the section (Zuber et al., 2005). As a consequence for the limiting membrane of the melanosome, it is only visible where the limiting membrane is aligned in the direction of the electron beam.

Another detail demonstrating the gain in resolution is shown by the cross-section of a microtubule (black arrow in Figure 4 and upper inset). As previously shown, CEMOVIS allows the distinction of single protofilaments (Zuber et al., 2005).

HPF samples for ET (electron tomography)

CEMOVIS provides structural preservation as close as possible to the native state together with a gain in resolution. It would be powerful to use CEMOVIS sections to solve the three-dimensional structure of subcellular compartments and macromolecular complexes by ET. However, ET of plastic embedded samples has already provided a lot of information on
Cryo-preparation of cells for transmission electron microscopy

Review

Figure 4 | Melanocytes
MNT-1 cells were frozen by HPF followed by FS and epon embedding (A) or processed for CEMOVIS (B) as described earlier (Hurbain et al., 2008; Couture-Tosi et al., 2010). (A) A stage III melanosome, with melanin deposits on the internal fibres. (B) The CEMOVIS image shows a stage III melanosome (III) and a cross-section of a microtubule (arrow). The lower inset shows a part of the melanosome in higher magnification with the limiting membrane (black arrowhead) and details of fibrils (white arrowhead). The upper inset is a higher magnification of the microtubule. Scale bars, 200 nm.

The three-dimensional organization of cells and will be first discussed below.

Principles of ET
Tilted series of thick sections of plastic-embedded samples or thinner sections of vitrified samples are generally acquired with a 200–300 kV electron microscope equipped with a eucentric tilt stage. The higher acceleration voltage allows the electron beam to penetrate the thick section, especially at high tilt angles (60–70°), where section thickness doubles or nearly triples. After acquisition, the single images of the tilt series are aligned and with the use of algorithms a three-dimensional image is produced.

ET of plastic-embedded sample
To collect images for ET, a grid with sections is placed in a high tilt holder and serial tilted views are collected, in general every degree over a +/−60° or +/−70° range with a CCD camera (charge-coupled-device camera). One of the limitations of ET is the impossibility of collecting a full range of tilted views (+/−90°). The information missing from the reconstruction is the so-called ‘missing wedge’. With dual-tilting schemes, the effect of missing data is reduced and resolution will become more isotropic (Mastronarde, 1997). The grid is rotated 90° and a second tilt series can then be imaged. During image acquisition, plastic-embedded biological samples can lose between 30 and 40% of their thickness due to radiation damage (Luther et al., 1988). To avoid this effect happening during acquisition, the specimen is exposed to the electron beam before data collection.

The reconstruction of an object from its two-dimensional projections is usually performed in two steps: alignment and then tomographic reconstruction. Tilt series can be aligned by fiducial markers such as small gold particles of 10–15 nm that are applied to the sections (Mastronarde, 1997; Amat et al., 2008). Alternatively, alignments without external fiducial markers use internal features of the sample that show a strong signal (Castano-Diez et al., 2010; Sorzano et al., 2009). For reconstruction of the tilt series different algorithms exist: the commonly used WBP (Weighted Back Projection; Radermacher, 1988) or more computationally consuming algorithms such as ART (Algebraic Reconstruction Technique; Herman, 1980) and SIRT (Simultaneous Iterative Reconstruction Technique; Gilbert, 1972). The latter offer more flexibility in the refinement of the reconstruction. After reconstruction of the tomogram, manual segmentation based on human vision and interpretation is often used to draw manually the contours of structures in different slices of an ET reconstruction. From those contours of the different slices three-dimensional models are created. As an alternative, tools for automatic segmentation and quantitative evaluation have been developed.
Different software packages for alignment, reconstruction, and segmentation exist and are in constant evolution (Jonic et al., 2008).

ET of plastic sections has resolved several subcellular organelles of mammalian cells such as the Golgi apparatus (Marsh et al., 2004), basal bodies (O’Toole et al., 2003), multivesicular bodies (Murk et al., 2003), melanosomes (Hurbain et al., 2008) and MHC class II compartments (van Nispen tot Pannewit et al., 2010). The ease of sectioning of plastic-embedded material allows tomography of serial sections, as well as of individual sections. In this way, the three-dimensional organization of large portions of cells or whole cells is resolved (Marsh, 2001; Höög and Antony, 2007). However, ET studies on HPF/FS-prepared samples are limited in resolution due to the presence of stain. Resolution should be increased with unstained images from sections of vitrified samples.

CETOVIS (cryo-electron tomography on vitreous sections)

Unlike the ET of stained plastic sections, CETOVIS of unstained cryo-sections has the potential to reach the resolution required for reliable interpretation at the molecular level by three-dimensional imaging in the near to native state of the cellular structures. However, tomogram resolution has been limited until now. The analysis of lysozyme crystals in two dimensions with CEMOVIS sections gave a subnanometre resolution, which was not achieved until now by CETOVIS because practical issues limit the theoretically possible resolution (Sader et al., 2009; Bouchet-Marquis and Hoenger, 2011).

The main restraint for CETOVIS is the low SNR (signal-to-noise ratio) of unstained sections. The cell contains a mixture of different structures and densities: doses that are high enough to result in good SNR can lead to specimen damage. On the other hand, doses that are low enough to preserve the specimen generate images that may be too noisy for useful three-dimensional reconstruction. In CETOVIS, a total dose of 100 e/Å² (1 Å = 0.1 nm) or less is commonly used. With the recent development of new, more efficient CCD cameras (FEI, Eindhoven, The Netherlands; Gatan, Pleasanton, CA, U.S.A.) and cryo-holders (Gatan) that permit the acquisition of reliable dual-tilt series, the SNR and resolution should increase.

The waviness of the section complicates the use of fiducial markers and different methods were developed to apply fiducial markers such as colloidal gold or quantum dot suspensions directly to frozen hydrated sections (Masich et al., 2006; Gruska et al., 2008).

Cutting artefacts of frozen hydrated sections need to be considered as well. The formation of crevasses limits the thickness of sections to 100–200 nm. When crevasses are present on the surface of the section, only the volume of the tomogram devoid of them is usable (Bouchet-Marquis and Hoenger, 2011). Compression can complicate interpretations of the corresponding three-dimensional reconstructions: this can be seen when the dimensions of globular structures such as ribosomes are measured along the cutting direction (Pierson et al., 2011). Whether compression has an influence on the structure of a macromolecular complex can be only seen by comparison with complementary techniques, such as X-ray data of the purified complex (Al-Amoudi et al., 2007; Pierson et al., 2010).

A strategy to increase the SNR and isotropic resolution in a CETOVIS tomogram is the averaging of extracted subvolumes from identical particles/complexes. This increases the resolution of the structure of interest either after purification or in situ (Nicastro et al., 2006; Al-Amoudi et al., 2007; Cope et al., 2010). A detailed description of this approach and possibilities of it has been given by Bartesaghi et al. (2008).

Conclusions

For biological samples rapid freezing represents a valuable tool to immobilize the sample. It acts simultaneously and avoids the leakage of ions and small molecules that occurs during chemical fixation at room temperature. After vitrification, a bulk sample with the need to be sectioned can be either processed by FS or frozen hydrated sectioning. Table 2 summarizes the main advantages and obstacles of both techniques.

FS offers improved morphological preservation compared with conventional preparation, which is due to the less harmful dehydration and, if added, the better controlled action of chemical fixatives. The stability of the resin-embedded sample after sectioning in the electron beam of the microscope makes this technique an excellent choice for ET studies.
CEMOVIS avoids any chemical modification of the sample and preserves the sample close to the native state. Compared with samples prepared by FS, CEMOVIS offers a higher resolution. The increased beam-sensitivity of the sections together with the difficulties to adhere the sections flat to the carbon film has made it a challenge until now to perform ET with CEMOVIS sections. A better understanding of the interaction between section and carbon film will lead to procedures that allow better adhesion of the section and increase the area to investigate.

To overcome cryo-sectioning artefacts, most notably compression, several groups investigated the use of focus ion beam systems. Here a Ga\(^+\) beam removes material from the sample, and it was important for any cryo approach to show that the interaction of the Ga\(^+\) ions with vitrified water during the milling did not heat the sample sufficiently to induce devitrification (Marko et al., 2006). With the ion beam the sample is either thinned (Marko et al., 2007; Rigort et al., 2010) or with the development of special equipment a section is prepared out of the vitrified samples (Hayles et al., 2010). It will be interesting to observe the further development of this approach.

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References
*Articles of special interest*


Fernández-Morán, H. (1952) Application of the ultrathin freezing sectioning technique to the study of cell structures with the electron microscope. Ark. Fys. 4, 471–491


Cryo-preparation of cells for transmission electron microscopy


Sartori, N., Richter, K. and Dubochet, J. (1993) Vitrification depth can be increased more than 10 fold by high pressure freezing. J. Microsc. 172, 55–61
Walther, P. and Ziegler, A. (2002) Freeze substitution of high-pressure frozen samples: the visibility of biological membranes is improved when the substitution medium contains water. J. Microsc. 208, 3–10

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MICROSCOPY SOCIETY OF AMERICA

CERTIFICATION BOARD

Practical Examination

Pledge of Independent Workmanship

Applicant’s Name:________________________________________________

Applicant’s Examination Number: ___________________________________________

I hereby state that all the procedures carried out in the preparation of the enclosed grids, microscope preparations and micrographs were performed exclusively by me and without any assistance.

Applicant’s Signature:____________________________________________________

Witness' Name (print): ______________ _____________________________________

Witness' Signature:  ________________ __________________________

Date:  ____________________________ _____________________________________

Location(s) where work was performed:
### BIOLOGICAL SCIENCE TISSUES (NONPATHOLOGICAL)

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**TOTAL SCORE**

Date:________
Grader's Signature:__________
MICROSCOPY SOCIETY OF AMERICA

Application for Certification
Electron Microscopy Technologist
Biological Transmission Electron Microscopy

Name: ______________________________________________________________________________________

Mailing Address: _____________________________________________________________________________

___________________________________________________________________________________________
___________________________________________________________________________________________

Is this address your residence? ______ Work? ______

Company/University (only if not part of above address): _____________________________________________

Daytime Phone: ( ____ ) _____________________     FAX: ( _____ ) ________________________________

E-mail address: _____________________________________________________________________________

I have read and understand the regulations pertaining to MSA Certification.

Your signature: ________________________________________________ Date: _______________________

EDUCATION (Start with High School)
School/Location/Years Attended Credit Hours Major Field Degree

___________________________________________________________________________________________
___________________________________________________________________________________________
___________________________________________________________________________________________

EMPLOYMENT (EM Related)

Current employer (name and address):

___________________________________________________________________________________________
___________________________________________________________________________________________
___________________________________________________________________________________________

Position/Title: Years employed there: ___________________________

Supervisor’s name: ________________________________
Previous employer (name and address):

_________________________________________________________________________________________
_________________________________________________________________________________________
_________________________________________________________________________________________

Position/Title: ___________________________   Years employed there: ________________

Supervisor's name: ____________________________________________________________