1 The role of scanning electron microscopy in cell and molecular biology: SEM basics, past accomplishments, and new frontiers

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1.1 Introduction

New developments in scanning electron microscopy (SEM) have resulted in a wealth of new applications for cell and molecular biology as well as related biological disciplines. New instrument developments coupled with new sample preparation techniques have been key factors in the increasing popularity of this versatile research tool. The desire to view biological material in native states at high resolution has stimulated new approaches to cryo-SEM applications and environmental SEM (ESEM). In addition, new staining techniques and novel processing applications that combine embedding and resin-extraction for imaging with high-resolution SEM has allowed new insights into structure–function relationships. Advances in immuno-labeling have further enabled the identification of specific molecules and their location within the cellular microenvironment. It is now possible to analyze macromolecular complexes within their three-dimensional cellular environment in near native states, and this in many cases has provided advantages over two-dimensional imaging with transmission electron microscopy (TEM). New instrument developments include helium ion microscopy that allows imaging greater details of cellular components; new technology approaches include automated block-face imaging combined with serial sectioning inside an SEM chamber that in recent years has increasingly been utilized for a variety of biological applications. Focused ion beam milling (FIB) combined with block-face SEM is among the newer approaches to analyze cellular structure in three dimensions. The present chapter introduces the basic features of SEM and sample processing for SEM, and highlights several advances in cell and molecular biology that have greatly benefited from using conventional, cryo-, immuno-, and high-resolution SEM.

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1.2 The SEM as a versatile instrument for biological applications

As has been highlighted in previous review papers and books, the SEM is known for its versatility, allowing imaging and analysis of large and small sample sizes and of a diversity of specimens in multiple biological disciplines. Numerous articles are available on SEM instrumentation, modes of operation, imaging capabilities, and resolution (reviewed in Pawley, 2008; Schatten, 2008, 2011); new books are also available that have addressed different aspects of SEM utilization (Schatten and Pawley, 2008; several others are reviewed by Hawkes, 2009). In addition, recent special topics issues of microscopy journals focused on SEM have been devoted to specific biological and material science applications, demonstrating the increased need for more specific information for the increased number of researchers applying SEM to biomedicine and the basic sciences. In this section, the SEM is briefly introduced and the importance of sample preparation for biomedicine and biology is highlighted and detailed for routine sample preparation as well as for several specific applications. Examples of sample preparations that have been designed for specific cellular and molecular investigations are presented in the individual chapters of this book.

For general information a schematic diagram, Figure 1.1, displays the basic components of a conventional SEM.

Images in the SEM are generated by probing the specimen with a focused high-energy beam of electrons that is scanned across the specimen in a raster scan pattern. The electron beam interacts with the specimen surface, and interaction of the beam electrons with the sample atoms produces signals that contain information about the specimen's surface topography and characteristic features. However, internal cellular structures can also be visualized by using preparation methods that "peel" off the regular surface layers and turn internal structures into surfaces that can then be viewed with SEM providing information on surface and internal structures of intracellular components. In addition, isolated cellular components can be visualized clearly by SEM. Such applications are included in Section 1.3 and are detailed for specific applications in several chapters of this book.

The incident electron beam interacting with the specimen produces emission of low-energy (<50 eV) secondary electrons (SE), back-scattered electrons (BSEs), light emission (cathodoluminescence), characteristic X-ray emission, specimen current, and transmitted electrons and others as displayed in Figure 1.2 (color plate). For routine SEM imaging an electron gun with a tungsten filament cathode or a lanthanum hexaboride (LaB_6) cathode is used while a field emission gun (FEG) is used for more detailed SEM imaging (reviewed by Pawley, 2008; Schatten, 2008, 2011). Specific detectors are used to generate information from the specimen: typically an Everhart–Thornley detector is used for SEs, a type of scintillation-photomultiplier system, while a dedicated detector of either a scintillation or semiconductor type is used for BSE detection. For routine SEM imaging a secondary electron detector is used for conventional imaging. This imaging mode may allow significant advantages over TEM, as the

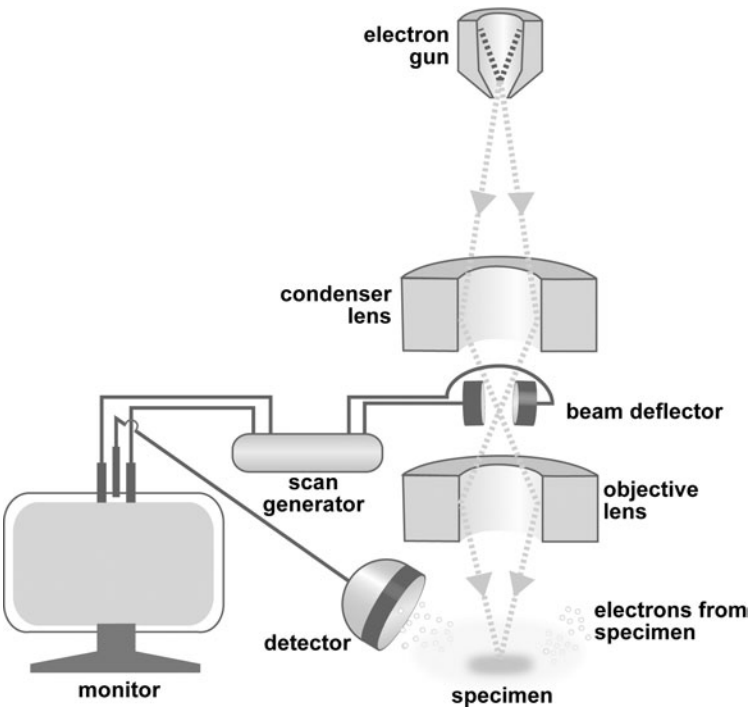

Figure 1.1 The basic components of a conventional SEM.

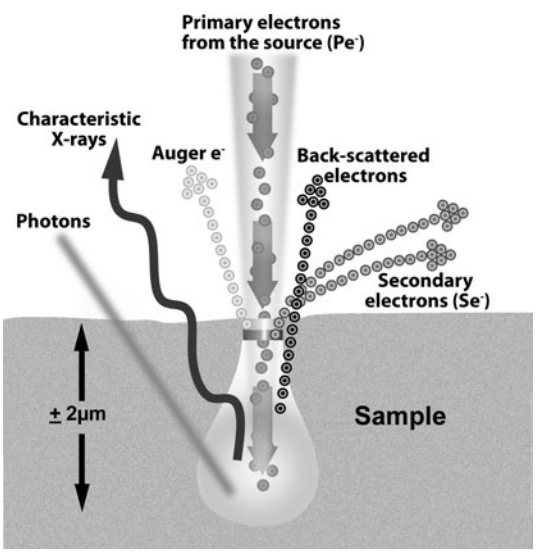

Figure 1.2 The incident electron beam interacting with the specimen produces emission of low-energy secondary electrons (SE), back-scattered electrons (BSEs), light emission (cathodoluminescence), characteristic X-ray emission, specimen current and transmitted electrons, and others as shown. (See plate section for color version.)

depth of field generates images that can readily be interpreted by the brain as three-dimensional representation. BSEs are beam electrons that are reflected from the sample by elastic scattering. The BSE signal intensity is related to the atomic number of the specimen and can therefore provide information about the different elements contained in the sample, which is oftentimes applied for imaging colloidal gold immunolabels of *c*. 5–10 nm in diameter. While characteristic X-rays for elemental analysis are used for biological applications, this form of analytical imaging is currently more frequently utilized in the material sciences to identify the composition of elements in a sample. However, new developments are in progress that may be amenable to biological applications and find new utilization in biology (Newbury, 2008). In this book, elemental analysis is described for the characterization of dental material (Dusevich *et al*., Chapter 13 of this book). Characteristic X-rays are emitted when an inner shell electron is removed from the sample by beam interaction, which causes a higher energy electron to fill the inner shell and release characteristic energy.

As discussed in detail in several chapters of this book, newer variations of SEMs include the ESEM that allows imaging of relatively unprocessed samples contained in low vacuum or gas. While this mode of imaging is not entirely practical for all biological samples it is excellent for biomaterials and several other biological samples as demonstrated by Dusevich *et al*. (Chapter 13 of this book). Most samples viewed in conventional SEM do require processing, which routinely includes chemical fixation with glutaraldehyde or formaldehyde to stabilize the specimen's mobile macromolecular structure by chemical cross-linking of proteins and osmium tetroxide to stabilize lipids. Cryofixation is being used to preserve structures in their close to native states, which can be achieved with liquid nitrogen or liquid helium temperatures, as described below and detailed in several chapters in this book.

For chemically fixed samples, dehydration follows to replace water with organic solvents such as ethanol (or acetone) in incremental steps that gradually include increased ratios of alcohol (or acetone) to water up to 100% alcohol steps. It is critically important to dehydrate samples fully without leaving water residues to avoid sample preparation artifacts. The preferred choice for sample drying is the critical point procedure, but there are alternatives if a critical point dryer is not available. The dried sample is then mounted on a specimen holder (also called specimen stub). A last step before sample analysis with SEM includes conductive coating of the sample to prevent accumulation of static electric fields which may be caused by electron irradiation during imaging.

1.3 General sample preparation for SEM

The four steps for sample preparation include a) fixation, b) dehydration, c) critical point drying, and d) coating. All four steps can vary significantly and require modifications for specific applications. Adequate sample preparation is critically important to maintain structural integrity and obtain reliable information on cellular components and molecular composition. Poor and inadequate specimen preparation undoubtedly causes artifacts and may yield wrong information, which in some cases has caused confusion and serious

concern in the literature (reviewed by Heuser, 2003). A great variety of sample preparation techniques and methods is available, and these have been elaborated by various investigators for specific research questions, some of which are presented in specific chapters of this book and others have been reviewed recently (Schatten, 2011). For routine SEM applications, the most common specimen preparation techniques are discussed below. Specialized sample preparation techniques are discussed in several of the other chapters of this book.

1.3.1 Conventional sample preparation

A most commonly used protocol includes fixing the biological sample with aldehydes such as 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer (PBS) followed by PBS rinses, post-fixation in 1% osmium tetroxide in 0.1% PBS, rinses in PBS, dehydration in increasing series of ethanol or acetone, critical point drying, mounting on aluminum stubs, and coating (reviewed in Schatten, 2011). The times for the specific protocol steps vary, depending on sample size and sample characteristics. For optimal results the investigator is referred to research papers in the specific area of interest or to specific *Methods* books series such as *Methods in Cell Biology* that contain detailed recipes with valuable notes sections addressing potential problems, hints, alternative approaches, and other most valuable information shared by researchers in their fields. General processing information for SEM is given below.

Chemical fixation is most frequently used for SEM sample preparation, but chemical fixation may destroy the structural integrity of certain structures or molecules of interest. For example, because glutaraldehyde cross-links the free amino groups of polypeptides and amino acids and inactivates enzymes it is inadequate for enzyme localization and related studies. In this case and several others, alternative preparation methods may yield better and more reliable results.

Dehydration is necessary to prepare samples for viewing in the SEM vacuum. As mentioned above for fixation, dehydration may be damaging to biological structures, as the shape of a macromolecule or membrane is produced and maintained by its interactions with water. This interaction may be destroyed by removing water during the dehydration process. Shrinkage of biological material has been observed and may need to be calculated for accurate measurements after preparing soft tissue for SEM (Boyde and Maconnachie, 1979, 1981). However, large macromolecules and their associated or covalently attached structural components are preserved and not affected by dehydration (Ris, 1985, 1988, 1990, 1991).

Critical point drying (CPD) is a most reliable method for drying samples in preparation for viewing in the SEM vacuum. However, several precautions are important to avoid artifacts (Ris, 1985), as traces of water contaminating the intermediate liquid used for drying (ethanol or acetone) or in the liquid CO_2 transition fluid may distort ultrastructure and induce considerable artifacts (Ris, 1985). Artifacts introduced by residual water after CPD had caused historic debates when new structures termed microtrabeculae (Porter and Stearns, 1981) were clearly identified as preparation artifacts (Ris, 1985; reviewed in Heuser, 2003). These studies and numerous others

have highlighted the importance of proper sample preparation as well as critical evaluation of instrumentation and accessory equipment. It further underlines the importance of critical sample evaluation and data interpretation. Thorough CPD is important for producing artifact-free specimens.

Coating of a specimen is an important aspect in sample preparation, as most biological structures have insulating characteristics and need to be made more conductive by applying a thin layer of metal to reduce the effects of charging (sample glaring; discussed in Hermann and Müller, 1991; Hermann *et al*., 1996). Heavy metals or heavy metal compounds are used as coating materials and include gold, gold/palladium, platinum, tungsten, graphite, and others (Walther, 2008) that are deposited either by high-vacuum evaporation or by low-vacuum sputter coating of the sample. Sample conductivity may also be increased by using the OTO (osmium, thiocarbohydrazide, osmium) staining method (Seligman *et al*., 1966; Malick *et al*., 1975; Familiari *et al*., Chapter 10 of this book). Determining the correct amount of coating that eliminates charging but allows reliable viewing of biological structures without obscuring the areas of interest is critically important.

Coating also improves contrast; coating thickness may vary with different samples (reviewed in Schatten, 2011).

Optimizing the coating thickness and applying specialized coating techniques (Walther, 2008) may be required for delicate biological structures, especially when viewing with low-voltage field emission SEM (LVFESEM) for which coating with 1–2 nm gold, palladium, or platinum applied by sputter-coating has been optimal for a large variety of samples (reviewed in Schatten, 2008). Coating may affect biologically relevant resolution (reviewed in Pawley, 2008). If charging problems are still encountered, alternative coating methods may need to be considered such as those developed by Walther and Hentschel (1989), who introduced a double coating technique by which the sample is first coated with a thin layer of heavy metal (platinum–carbon or tungsten with an average thickness of 2–3 nm) followed by a 5–10 nm carbon layer (Walther, 2008). Specific coating procedures have been described and discussed in the literature (Peters, 1980; 1982; 1985; 1986a; 1986; 1988; Peters and Fox, 1990; Walther, 2008; reviewed in Schatten, 2008), which includes Pt, W, and Ta by DC-ion sputtering to view cells growing on EM grids (Lindroth *et al*., 1988; Bell *et al*., 1989; Lindroth and Sundgren, 1989).

For optimal analysis of cellular and molecular components, appropriate sample preparation and the investigator's expertise with specific biological samples are among the most important criteria for reliable results using SEM. More difficult preparations are oftentimes encountered with plant samples or microbiology specimens including bacteria or parasites for which new preparation techniques may need to be developed. An example for the development of such techniques has been described for *Toxoplasma* (Schatten and Ris, 2002, 2004; Schatten *et al*., 2003). In *Toxoplasma*, actin visualization had been a problem and several hurdles had to be overcome through step-wise and complementary approaches to determine localization of the fixation-sensitive actin-like fibers. Dobrowolski *et al*. (1997) had used cryo-fixation to determine immunolocalization of actin molecules, which established the presence of actin immunogenicity

underneath the *Toxoplasma* surface. Subsequent experiments were performed peeling off the outer surface layer by quick treatment with detergent followed by cytoskeletal stabilization and fixation that revealed actin-like fibers underneath the surface (Schatten *et al*., 2003). This example demonstrates that specific biological expertise is important to reveal structure–function relationships using SEM; new preparation methods may need to be designed paying attention to the specific biological characteristics and dynamics that require specific biological knowledge to preserve delicate structures that may respond differently to different chemicals used in the preparation protocols. The importance of biological expertise to obtain optimal information is demonstrated in numerous examples. The different requirements to preserve different structures reliably had already been recognized in the early pioneer days of electron microscopy when most samples were fixed in the cold, unknowingly destroying the cold-sensitive microtubule fibers. Correlative microscopy (also see Albrecht *et al*., Chapter 6 of this book) is oftentimes needed to obtain accurate information for biological material. When it was recognized that cold-fixation indeed destroyed microtubules and the debate was settled, other debates emerged that questioned the characteristics of microtubules that in cross-sectioned EM samples were featured as short stubs, while immunofluorescence microscopy with anti-tubulin antibodies revealed long microtubules that had not previously been shown with TEM. These historic examples clearly show that sample preparation and interpretation of results are highly important and may require several approaches for reliable identification of biological material.

For plant material, optimal SEM preparation techniques are still being elaborated, as plant cells are more difficult to prepare for SEM because of tissue rigidity resulting from polysaccharide-containing cell walls and the large vacuole spaces within cells (Cox *et al*., 2008). In several cases, use of protoplasts has been the choice for plant material, as protoplasts can be analyzed after removal of the cell wall containing large amounts of cellulose that hinders optimal processing. Isolation and detergent extractions of plant material have resulted in stunning data for cellular components, as seen in Chapter 9 of this book by Schroeder-Reiter and Wanner, which displays details of plant chromatin. Specific processing for microorganisms has been described in excellent detail by Erlandsen (2008).

1.3.2 Freezing methods

Ultra-rapid freezing is frequently used to preserve molecules in a more native state compared to chemical fixation. Ultra-rapid freezing demands avoiding the formation of damaging ice crystals in cells or tissue, which is accomplished by freezing at a rate of 10^4–10^5 degrees C/second. At this cooling rate, cellular water becomes vitrified rather than forming ice crystals. Several freezing methods are readily available, some of which are described in the specific chapters of this book. The basic freezing methods include the following:

Plunge freezing allows an average depth of vitrification of *c*. 1–2 μm with minimal ice crystal artifacts, which is achieved by plunging a specimen into a liquid cryogen such as supercooled liquid nitrogen or supercooled ethane or propane.

Slam freezing (cold metal block freezing) allows an average depth of vitrification of *c*. 10–15 µm with minimal ice crystal artifacts, which is achieved by slamming a specimen onto a copper or silver block that has been chilled to −196 to −269 °C with liquid nitrogen or liquid helium.

Propane jet freezing allows an average depth of vitrification of *c*. 40 µm with minimal ice crystal artifacts, which is achieved by sandwiching a 200–500 µm thick specimen between two metal plates that are clamped into a device that directs jets of liquid propane cooled with liquid nitrogen against both sides of the specimen plates.

High-pressure freezing allows an average depth of vitrification of *c*. 500 µm with minimal ice crystal artifacts. This modification of propane jet freezing or liquid nitrogen freezing is achieved by pressurizing the specimen to 2100 atmospheres to suppress or reduce growth of ice crystals at the moderate freezing rates that can be achieved in the depth of the sample. High pressure lowers the freezing point of water as well as the rate of ice crystal formation.

These basic freezing methods and several modifications have been applied with great success to a variety of specimens (reviewed in Schatten, 2011). Freezing followed by freeze drying is among the methods of choice for many applications in cell biology (Pawley and Ris, 1987) and freezing followed by freeze-substitution has gained increasing popularity (Erlandsen, 2008) for the superior ultrastructural preservation of cellular components and structures. Direct observation of frozen specimens (Pawley *et al*., 1991) has provided resolution above 3 nm, and freeze–fracture (Haggis and Pawley, 1988) has been applied successfully to visualize and analyze intracellular structures (reviewed in Pawley, 2008). Analysis of intracellular structure has further been accomplished by dry fracture of tissue culture cells achieved by touching intact cells to the surface of adhesive tape (Lim *et al*., 1987; Ris, 1988, 1989; Ris and Pawley, 1989; Sepsenwol, Chapter 3 of this book), allowing excellent insights into intracellular structure. Viewing of incorporated labels that decorate internal cell structure can also be accomplished with this method.

Among the advantages of cryofixation over chemical fixation is the arrest of cells in a "life-like" state; cryo-immobilization takes only milliseconds compared to chemical fixation, which may take seconds, or even longer. Freeze-substitution in acetone, methanol, or other solvents is frequently used for subsequent processing and permanent fixation.

In addition to freezing alone, several combination methods have also been used for specific biological applications including cryo-SEM of chemically fixed cells, as described by Erlandsen (2008). Other investigators have used chemical fixation with very low concentrations of glutaraldehyde (0.1–1.0%) for 10 to 15 min to stabilize macromolecules prior to cryo-immobilization (Centonze and Chen, 1995; Chen *et al*., 1995). Such fixation approaches preserved macromolecular complexes excellently and revealed remarkable detail of actin filaments (Erlandsen, 2008) after coating with chromium, allowing clear visualization of the helical twists of two polypeptide chains in the filament and 5 nm subunits (reviewed in Schatten, 2011). In other studies using

cryo-methods, biological resolution of 2–3 nm could be achieved when viewing macromolecular complexes with high-resolution in-lens cryo-SEM (Erlandsen *et al*., 2001), revealing details of the glycocalyx on the extracellular surface of human platelets that were labeled with three colloidal-gold markers to detect all three cell-adhesion molecules in the glycocalyx. This study used plunge-freezing into propane chilled with liquid nitrogen followed by partial freeze-drying at −85 °C and the double-coating method developed by Walther *et al*. (1995). This coating method involves cryo-coating by evaporation of 2 nm TaW at 45° through electron-beam deposition and 7–10 nm carbon at 90 °C. The double-coating technique has also been used in numerous other applications including double-layer coating of yeast cells after high-pressure freezing and freeze–fracture (reviewed in Erlandsen, 2008). All these examples clearly demonstrate that combination methods of various complexities and high-resolution SEM coupled with specific expertise of biological structure can be superior over other imaging methods and can reveal unique three-dimensional information.

If an SEM is equipped with a cold stage for cryo-microscopy, additional preparation techniques are available including cryo-fracture under vacuum, sputter coating and transfer to the SEM cryo-stage while still frozen. This method is particularly useful for imaging and analysis of temperature-sensitive cellular components including fats. Direct viewing of frozen specimens in the SEM by using a cold stage had become possible when side-entry eucentric goniometer stages were developed that could accept high-stability, cryotransfer stage rods. Uncoated and slightly coated specimens have been viewed in this mode (Herter, 1991; Pawley *et al*., 1991; Müller *et al*., 1992; Boyde, 2008; Walther, 2008), allowing detailed three-dimensional information to be obtained, as for example by stereo-imaging of frozen-hydrated mitochondria. Combination methods include freeze–fracture followed by the thaw–fix technique developed by Haggis (Haggis, 1987; Haggis and Pawley, 1988), involving fresh-freezing in propane, freeze–fracture and thawing into fixative, critical point drying and ion-beam-sputter coating with Pt before imaging. These examples also demonstrate the usefulness of stereo-imaging that allows better understanding of complex structural interactions of cellular components (reviewed in Pawley, 2008).

1.4 High-resolution low-voltage SEM and combination methods

Several chapters in this book describe use of high-resolution low-voltage field emission SEM (HRLVFESEM) with great success to view and analyze isolated structures or delicate internal cellular components. These applications have greatly benefited from the development of field-emission sources that has allowed formation of an intense beam of low-voltage electrons with small beam diameter (reviewed in Albrecht and Meyer, 2008).

HRLVFESEM has increasingly found new applications in cell and molecular biology for the study of structure–function relationships on three-dimensional levels. In addition, HRLVFESEM has also been an indispensable approach to image and analyze isolated structures that previously had been analyzed mainly by TEM negative staining. These new applications utilizing low-voltage electron microscopy take advantage of accelerating voltages at or below 5 keV. With these new capabilities combined with improved sample

processing and sample coating as described in several chapters of this book, modern SEMs can achieve resolution for biological material down to 2–5 nm, a level previously only possible with TEM (reviewed in Schatten, 2011). As shown in subsequent chapters in this book, detailed analysis of chromosomes, cytoskeletal components, viruses, and other biological material has been performed using HRLVFESEM and revealed new detailed information in three dimensions that is superior to data obtained with TEM.

New applications with cryo-SEM including cryo-microtomy of cryo-immobilized plant and animal cells (Nusse and Van Aelsi, 1999; Walther and Müller, 1999) have yielded new information on internal cellular structures. In these specific applications the surface of the tissue block is examined rather than sections by cryo-SEM. Furthermore, a cryo-dual beam instrument has been utilized that incorporates both focusing electrons (SEM) and focusing ion beam (FIB) columns. Such applications have been employed by Mulders (2003) to analyze biological samples including yeast, bacteria, and gut epithelial cells.

New combination methods have been developed that will be addressed below. These new applications and others offer new approaches to identify biological components reliably inside cells, as described in excellent detail in several chapters of this book. A variety of different methods may need to be employed for optimal information, taking into consideration that some biological specimens are more fragile and complex than others, requiring more complex specimen preparation and processing.

In the author's lab, FESEM has been used to image delicate mitotic spindles and sperm asters yielding new information on cytoskeletal interactions in three dimensions (reviewed in Pawley, 2008; Schatten, 2008, 2011). In addition, the technique has allowed analysis of isolated centrosomes in three-dimensional configuration (Thompson-Coffe *et al*., 1996).

1.5 New developments and future perspectives

One of the goals for new instrument and sample preparation development is to achieve higher resolution and imaging of samples in more native states. Such approaches have been pursued in recent years by Boyde (2008) and by researchers designing various types of microfluidic chamber that can be placed inside an SEM (Thiberge *et al*., 2004; Boyde, 2008). The design and testing of microfluidic chambers is intensively being pursued by several groups with applications for transmission EM and SEM (Thiberge *et al*., 2004; Klein *et al*., 2011).

A most impressive development first presented by Denk and Horstmann (2004) introduced automated block face imaging combined with serial sectioning inside the chamber of an SEM. This development required several technical modifications that have been described in detail (Denk and Horstmann, 2004). With this new technology development the authors were able to trace even the thinnest axons to identify synapses in nerve tissue. The authors reported several hundred sections of 50–70 nm thickness that will lead to further developments to reconstruct large areas of neuronal tissue. Building on these developments, Knott *et al*. employed combination methods using light and electron microscopy. Serial section SEM of adult brain tissue using focused ion beam milling allowed visualization of the ultrastructure (Knott *et al*., 2008). Several