Accessing Nuclear Structure for Field Emission, in Lens, Scanning Electron Microscopy (FEISEM)

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ACCESSING NUCLEAR STRUCTURE FOR FIELD EMISSION, IN LENS, SCANNING ELECTRON MICROSCOPY (FEISEM)


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Abstract

Scanning electron microscopy (SEM) has had a shorter time course in biology than conventional transmission electron microscopy (TEM) but has nevertheless produced a wealth of images that have significantly complemented our perception of biological structure and function from TEM information. By its nature, SEM is a surface imaging technology, and its impact at the subcellular level has been restricted by the considerably reduced resolution in conventional SEM in comparison to TEM. This restriction has been removed by the recent advent of high-brightness sources used in lensfield emission instruments (FEISEM) which have produced resolution of around 1 nanometre, which is not usually a limiting figure for biological material.

This communication reviews our findings in the use of FEISEM in the imaging of nuclear surfaces, then associated structures, such as nuclear pore complexes, and the relationships of these structures with cytoplasmic and nucleoplasmic elements. High resolution SEM allows the structurally orientated cell biologist to visualise, directly and in three dimensions, subcellular structure and its modulation with a view to understanding its functional significance. Clearly, intracellular surfaces require separation from surrounding structural elements in vivo to allow surface imaging, and we review a combination of biochemical and mechanical isolation methods for nuclear surfaces.

Key Words: nucleus, nuclear envelope, nuclear pore-complex, nucleocytoplasmic transport, nucleoplasmmin, field emission in lens scanning electron microscopy, chromium coating, colloidal gold, backscatter electron imaging.

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Introduction

Current studies on the nucleus by a variety of novel microscopic techniques are producing something of a renaissance in our understanding of nuclear structure and function. The organisation of peripheral structure, namely the nuclear envelope and nuclear pore complexes [3, 7, 15-19, 14, 25, 29-31, 36, 41, 42]; the internal organisation with respect to subjects such as laminas [1, 14, 18, 19, 20, 21, 24], coiled bodies [11], transcriptional activity [38, 39] and chromosomes [32,38] have all shown striking progress over the last few years. Nuclear transport [2, 10, 11, 12, 13, 22, 23, 26, 29] is under active investigation, both biochemically and structurally: in vitro systems have produced significant progress in accessing the dynamics of mitosis [18, 40], and the control, mechanics and role of the nucleus in programmed cell death (apoptosis) is also being addressed [35]. This communication to reviews our own recent attempts to visualise, at macromolecular resolution, directly and in 3D, the organisation of the nuclear envelope and associated structures [4-8, 17, 18, 34] using field emission, in lens scanning electron microscopy (FEISEM) supported by conventional transmission electron microscopy (CTEM).

FEISEM, well established with electron microscopists, has only relatively recently begun to realise its potential in cell biology, perhaps as a result of relatively limited accessibility to investigators in this field. Field emission instruments with specification resolutions of around 1 nanometre, provide essentially the same effective resolution in biological material which has been available in transmission electron microscopy (TEM) for the past 25 years. Whereas conventional TEM (CTEM) tends to be restricted to relatively thin (30-60 nm) sections, or isolated molecules spread thinly on a support film, (with the exception of high voltage electron microscopy (HVEM)) the advantage of field emission in scanning instruments is that specimens of infinite depth can be surface imaged to much the same effective resolution as TEM. Two criteria must be fulfilled to achieve TEM resolution in a scanning electron microscope (SEM) - firstly a high brightness source, allowing
sufficient signal to be collected by rastering the specimen with a very small spot size (0.7 to 1.0 nm diameter), secondly efficient collection of that signal to provide relatively noise free images at 200 to 300 thousand times magnification. The high brightness criterion has been met by the production of field emission (FE) sources, which fall into 2 categories, namely cold FE, or thermally assisted (‘Schottky’) sources, which vary in their properties and requirements. Cold field emission, as its name suggests, works at low temperatures, but to overcome the ‘work function’ required to produce field emission in these conditions from a pointed tungsten tip, requires $10^8$ pascals operating pressure in the gun region, and regular ‘flashing’ of the tip to disperse contamination on the tip surface, which significantly reduces emission. Cold field emission is inherently unstable, but this has been overcome as an operating difficulty by a sophisticated feedback loop to the photomultiplier.

Thermally assisted, or ‘Schottky’ field emission maintains the tip at around 1800°C, repelling any potential contamination, and obviating the requirement for flashing. In combination with doping the tip with Zirconium oxide, the higher temperature allows the work function for field emission to be overcome at 2 orders of magnitude lower vacuum ($10^6$ pascal) in the gun area. Schottky emission is stable, and is consequently the FE tip of choice for beam writing and microanalysis. In terms of absolute resolution, the virtual source size at the point of emission from cold FE sources is smaller than the Schottky, but this seems to produce very little difference in performance by the time the beam hits the specimen. In general, costs of production of Schottky systems are cheaper than cold FE systems, and Schottky tips may well exceed 10,000 hrs of consistent emission.

The second requirement for achievement of 1 nm resolution has been the positioning of the specimen in the electron optical column in the field of the final lens (like conventional TEM), where spherical and chromatic aberrations are at their minimum, and also the secondary electron emission from the specimen surface is effectively ‘trapped’ in the magnetic field of the lens, allowing a very high efficiency of collection from the ‘in lens’ detector. More recent FE instruments however have come close to the same resolution using close (3 mm) working distances in a conventional ‘pinhole’ final lens arrangement.

Both cold FE and Schottky sources allow good resolution (around 4 nm) at very low (1.0-1.5 kV) accelerating voltages, allowing surface imaging with minimal penetration and beam damage. However, this is still short by a considerable amount of the 1 nm resolution achievable at around 30 kV, which has been aided by the current developments in thin coatings (1.5-3 nm) with refractory metals (eg. chromium, tungsten and tantalum). Thus several types of FE instruments have a performance which in general cannot be considered limiting for biological specimens, leading to the continual quest for optimal preservation procedures, but with enormous potential for direct, macromolecular three-dimensional (3D) imaging of the surface of bulk specimens.

Observation of the surface of an internal membrane bound structure such as the nucleus, clearly requires direct access by the electron beam. Once this has been achieved, the ability to view structure on that surface is not limited by section thickness, nor uncertainty of plane of section and limited contrast dependent on electron dense staining (as in TEM), and a ‘panorama’ of fresh surface detail becomes apparent. This was graphically demonstrated by Hans Ris [36, 37] in his description of the asymmetry of the nuclear pore complex on either face of the nuclear envelope, for 30 yrs assumed identical on the available TEM evidence. This evidence also included the excellent freeze fracture studies which established much of what we know of nuclear pore complex numbers, distribution and dynamics, but because of the nature of a frozen fracture to split pore complexes at the planes of the nuclear membrane, could not observe peripheral structures. As Ris [36] pointed out in his original article, the nuclear pore complex is particularly unsuited to either thin section or whole mount observation. Due to its size (110 nm in diameter, 80-100 nm high) bulk (estimated mol.wt. 120 million Da) any thin section (30-60 nm) will only present a portion of the structure, and the contrast imparted by negative staining of whole mounts is also limited, as 80 nm depth of negative stain would be impenetrable by TEM. More recently however, we must allow for the ‘thin ice’ approach coupled to powerful computerised image reconstruction from minimal contrast, but this approach still produces an ‘averaged’ structure, which in the case of nuclear pore complexes (NPCs) involved in dynamic transport processes, may ‘average out’ crucial structural variations related to different functional states.

Accessing the Nucleus for Surface Imaging

In choosing to partition its genetic material (unlike bacteria) the eukaryotic cell (presumably unwittingly) created many difficulties by the separation of the nucleus and cytoplasm, mainly in the provision of efficient communication and transport between them. This giant evolutionary leap also led to the frustration of future investigators finding the surface of the nucleus in which they were interested to be firmly embedded in cytoplasm. The approaches taken to get round this problem
fall into two categories, nuclear isolation and nuclear exposure.

**Nuclear isolation of large nuclei**

This involves wholesale removal of the nucleus from the rest of the cell, a process clearly involving disruption of the cell membrane, dispersion of the cytoplasm, and the exposure of a 'clean' nuclear surface. This is all clearly less than physiologically optimal, and as the nucleus may well have peripheral cytoplasmic attachments, will involve a separation of any links close to its surface. Care should also be taken with the milieu into which the nucleus is isolated. Using amphibian material, isolation buffers (based on unbuffered 5:1 mixtures of 0.1 M KCl and 0.1 M NaCl, pH 6.0-7.5) will be varied with different and amphibian species. With insect polytene nuclei, isolation is performed in insect Ringers solution (7.5 g NaCl, 0.35 g KCl and 0.21 g CaCl₂ [27]. Clearly, once elements of the cell are isolated, they should be maintained in as near physiological conditions as possible. [5, 27]. Manual removal of part of a cell is made considerably easier the bigger the cell is. For this reason, the amphibian oocyte (usually *Xenopus* which is easily isolated from 'parental' tissue) has become the cell of choice for much nuclear investigation. Viewed with a bench stereomicroscope and maintained in isolation buffer [5, 27] the oocyte membrane is slit with a fine needle, the yolk squeezed through the slit, and the nucleus (0.2 mm in diameter - within the resolution of the unaided eye) is recognised as a 'pearly' sphere during its exit, and can then be washed using pasteur pipettes and its contents (lampbrush chromosomes) dispersed, and the 'empty' nuclear envelope utilised for observation. In our case, the envelope is deliberately ripped as it is spread onto the surface of a 5 mm square silicon chip, to which it adheres naturally in much the same way as it would to a glass cover slip. The advantage of a silicon substratum (although opaque to transmitted light) is that because it is a good electrical conductor (unlike glass) it helps to facilitate imaging at 30 kV acceleration voltage in the FEISEM (Fig. 1). Once attached and spread - allowing observation of both nucleoplasmic and cytoplasmic sides of the nuclear envelope (NE), the preparation can be extensively washed (20-60 mins in isolation buffer) then fixed, or alternatively structurally modulated by proteolytic or detergent extraction, or labelled by immunocytochemistry prior to fixation. Fixation of a relatively fragmentary piece of tissue is usually 10 mins in 2.0% glutaraldehyde (possibly with added tannic acid (0.2%), 10 mins 0.1% OsO₄ (both in isolation buffer [4, 15]) followed by 1% aqueous uranyl acetate for 10 mins. This is followed by dehydration through an ethanol series, and critical point drying (CPD) from high purity CO₂ using 'Arklone' (Arcton 112, ICI Chemicals, Runcorn, UK) as transitional fluid. 100% ethanol and arklone are stored over molecular sieve to facilitate absolute dehydration, and the 99.999% pure CO₂ (H₂O < 5 ppm) passed through a molecular filter (Tousimis, Rockville, MD, USA). Critical point drying, was performed in a Balzer's (Liechtenstein) CPD unit, and the chips were transferred directly from the CPD chamber to the coating unit (Edwards Auto 306, Crawley, U.K.) where they were pumped to 5x 10⁻⁸ using a cryopumped system. Once this vacuum was achieved (40-50 mins) the system was backfilled with high purity argon to a pressure of 75 x 10⁻³ mbar, and sputter coated with either chromium or tantalum to a thickness of 1.5 to 2.0 nm as measured by thin film monitoring at a deposition rate of around 0.1 nm/sec. Oxidation of the target was removed by 30 secs pre-sputtering with a shutter covering the specimens. During coating the specimens were rotated at 150 rpm at an angle of 45° to the target. Wherever possible, specimens were examined as soon as possible after coating. Despite the fact that chromium and tantalum coatings can provide good signal for a few days to a few weeks, there is no doubt that optimum imaging occurs soon after coating. The post-fixation stages of this protocol can be considered constant for all subsequent preparations, unless stated.

Manual isolation of nuclei from other oocytes has also been applied to a variety of other species. Avian oocytes from both Pigeon and Chick have generated successful NE preps, as have polytene nuclei from insect salivary glands such as *Chironomus* [7], establishing the nuclear pore basket as an evolutionarily conserved structure. As nuclei get progressively smaller (eg. *Chironomus* polytene nuclei - approx. 50 µm in diameter) the manual isolation of nuclear envelopes demands a large increase in skill (and patience!)

**Isolation of small nuclei**

Mammalian nuclei from tissue culture cells such as CHO (Chinese Hamster Ovary) rarely exceed 10 µm in diameter, and although they are routinely microinjected and can be considered manipulable to some degree, they really fall below the lower limit for manual isolation. Because of their small size, working 'one at a time' is clearly precluded, and consequently it is best to start with large numbers (millions) to achieve reasonable numbers on each preparation, ideally around 10,000 per chip. Thus the approach has a biochemical, as well as a mechanical element. A pellet of suspension grown tissue culture cells (eg. K562) is resuspended in a hypotonic buffer designed to permeabilise the cell membrane and swell the cytoplasm, with the actual 'release' of nuclei achieved by 2-3 strokes of the suspension between the sides of a test tube and plunger (Dounce Homogeniser)
creating a shearing force which disrupts the cell membrane and cytoplasm, releasing the nucleus into suspension [34]. At this stage the nuclear surface retains an extensive coating of cytoplasm and endoplasmic reticulum which is then removed by spinning through a sucrose gradient, ‘cleaning’ the nuclear surface. In our hands the osmotic consequences of exposure to sucrose for unfixed nuclei were not acceptable, and consequently percoll was found to be a considerable improvement [30]. Nuclei were isolated from the correct part of the gradient, resuspended in a physiological buffer, [34] and spun onto poly-l-lysine coated silicon chips, fixed and dehydrated, critical point dried, and coated as above, but in this case with 5-6 nm Cr, as charging was more apparent at 30 kV on spherical nuclei than on spread NEs on silicon chips. In these preparations good structural preservation of NE and NPCs was achieved (Fig. 2), and surface visualisation of apoptotic clustering of NPCs was observed [34, 35].

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**Figure 1:** Low magnification image (x10 in instrument) (30 kV accelerating voltage) of area of 5 mm² silicon chip used for mounting of biological specimens. This particular chip has been used in dry fracture and shows adhesive and fractured nuclei. (Bar=2 nm).

**Figure 2:** Nucleus from human cell line (K562) after detergent free isolation and purification on an isotonically balanced Percoll gradient showing the surface of the NE and NPC structure. (Bar=100 nm).

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**Exposure of the nuclear surface in situ**

This methodology was evolved to retain the structural polarity of nucleus and cytoplasm, for tissue culture cells grown attached to the substratum rather than in suspension culture (above). As with suspension cells however, the membrane must be permeabilised and cytoplasm ‘diluted’ before the nucleus can be exposed, in this situation after fixation, in contrast to the previous method. This process involves a brief ‘stabilisation’ fixation of short duration and low strength fixative, followed by extraction with detergent. These processes are related, the shorter the fixation consistent with NE stabilisation, the shorter the subsequent detergent extraction is required. We have found this to be variable for different cell lines, but in the case of *Xenopus* fibroblasts grown in tissue culture, prefixation with 1.0% paraformaldehyde and 0.01% glutaraldehyde in phosphate buffered saline (PBS) for 1 minute required 1 - 3 hrs extraction with 0.5% Triton X-100 (also in PBS). For mammalian tissue culture cells extraction needed to
Accessing nuclear structure for FEISEM

Figure 3: Surface view of *Xenopus* tissue culture cell nucleus after dry fracture subsequent to the protocol described in text. Two areas of nuclear envelope, either side of exposed chromatin, (centre) display nuclear pore complexes, (arrowed) and remnant cytoskeletal filaments (arrowheads). Differential packing of the chromatin filaments is also apparent. (Bar=500 nm).

be longer, 4 - 6 hrs (but this can be checked by observing decreasing birefringence using phase contrast microscopy) the danger being that an extended extraction will cause the cells to detach from the substratum.

After prefixation and extraction, the extracted cells are re-fixed normally in 2% glutaraldehyde followed by OsO₄ (sometimes with one OsO₄-thiocarbahydrazide-OsO₄ rotation) for 20 - 30 minutes each. This is followed by conventional dehydration and CPD. At this stage the cover slip (or Si chip) on which the cells were grown is touched to double-sided adhesive (Agar ‘Sticky Tabs’, Agar, Stansted, UK) and separated without 'sliding' the surfaces against each other. Both the original cells and the chip with adhesive should be retained as both carry fractured surfaces. These are coated normally (1-2 nm chromium) and observed by FEISEM. In most cases, despite 'firm' contact with the adhesive, there are only scattered patches of cells which have fractured, but these are easily found. Separation through the cell can occur at all planes, but nuclear surfaces, displaying a similar quality of preservation to isolated unfixed nuclei [14, 19] are regularly observed, often with adjacent adherent cytoskeletal elements, possibly indicative of NPC-cytoskeleton attachment. Although regular exposure of the nuclear envelope surface occurs with this type of fracture, the planes of fracture are not limited to following membranous 'planes of weakness' as occurs in frozen fracture [28]. In the same cell, a whole series of step-like fractures across upper nuclear surface, through several levels of nuclear contents, through the underside nuclear membrane and into the cytoskeletal remnants of the cytoplasm is a common event (Fig. 3). This allows 3D imaging of chromatin packaging *in situ* and does present the relatively 'loose' and tight packaging in surface imaging of fibre organisation that would be envisaged from the electron lucent and electron dense regions of euchromatin and heterochromatin respectively in TEM thin sections. Nucleoli appear to be too dense for fractures to occur through their dense cored regions, so that the fractures in nucleolar areas tend to leave them as a spherical 'outcrop' also indicating some sort of interfa-
Figure 4: Deresined section of HeLa cell after detergent extraction, and extraction of nuclear DNA showing the remaining nucleoskeletal structure, which also has prominent nucleoli (N). (Bar=2.0 µm).

Special separation between the nucleolar surface and surrounding nucleoplasm.

Exposure of 3D Nuclear Structure in Deresined Sections

This technology allows the use of resin embedded material to generate specimens for 3D imaging, by accessing the area of interest by exposure at the face of a cut section, and removal of resin embedding exposing 3D detail within the depth of the specimens, which are usually cut in the region of 200-500 µm thick. To observe ‘whole’ fixed and embedded material in this way is usually limited, as resin removal produces little surface topography, on account of the overall fixation of the cell contents. If however, the specimens have been extracted, removing or retaining specific structures, then they may well be imaged to advantage to complement the transmitted image produced in the TEM [9]. In the case of nucleoskeletal preparations for example, the DNA and associated proteins are removed from the nucleus using endonucleases to cleave the DNA, followed by removal of the fragments by electroelution, starting from small numbers of cells encapsulated in agar beads [21] which protect their biochemical functions during cytoplasmic and nuclear extraction. Structures remaining in these preparations are the cytoplasmic cytoskeletal filaments, nuclear envelope/pore complex residues, and the nucleoskeleton itself, organised as a network of filaments of different sizes [23], clearly attached to the peripheral remnants of the nuclear envelope at one end, and often attached to retained nuclear structures such as the nucleolus, at the other (Fig. 4). FEISEM imaging of these preps gives some idea of the texture of the nucleolar e-dense regions too electron opaque for TEM, and allows individual fibres to be followed over relatively long distances within the

Figure 5: Early stages of in vitro nuclear formation, showing Xenopus demembranated sperm head surface, with binding of numerous vesicles from the cytoplasmic extract to the surface of the sperm chromatin. (Bar= 100 nm).
thickness of the deresined section. Backscattered electron imaging of colloidal gold labelling to areas of DNA replication also has advantages in visualising gold colloid in sites of high electron density that would not be visualised in transmitted electron images.

In Vitro Nuclear Formation

This cell free system obviates the need for separation of the nucleus from its surrounding cytoplasm, as it relies on the properties of amphibian or mammalian cytoplasmic extracts to reform functional nuclear structure in vitro. Briefly, demembranated sperm heads from *Xenopus* are incubated in cytoplasmic extracts from *Xenopus* eggs, in the presence of an adenosine triphosphate (ATP) regenerating system. Nuclear formation takes place at room temperature over 1 - 2 hrs [40].

Initially the sperm chromatin is decondensed at the surface, and binds numerous vesicles present in the extract, which flatten, fuse and form a new, complete nuclear envelope over a period of around 1 hr [18] (Fig. 5). At the same time nuclear pore complexes are formed in the NE, and once NE formation is complete, exact DNA replication takes place. This is dependent upon, and controlled by the nuclear envelope, which ends replication at the exact duplication of the DNA. Experimental permeabilisation of the nuclear envelope at this stage will result in a further blip of DNA replication. In some extracts, replication is followed by chromatin condensation akin to the early stages of mitosis. The breakdown of completed nuclei can however be routinely stimulated by addition of recombinant cyclin B protein to interphase extracts to activate the universal mitotic kinase p34cdc2 which generates an in vitro mitotic breakdown of pre-formed nuclei. Clearly, this is an extremely attractive system for surface imaging of the interactions between forming NE and chromatin stages in NPC formation, and the processes occurring at mitotic breakdown. All that is required prior to fixation is a gentle resuspension of the extract, washing away excess material at the nuclear surface, spinning down onto Si chips, and fixation in situ, allowing comparative observations of hundreds of nuclei in each of a series of time course fixations separated if necessary by no more than 60 secs. One further advantage of the system is the scope for fractionation of the components of the extracts, and reformation in the absence of specific components by depleting the extract using antibodies linked to magnetic beads [20]. FEISEM allows 3D imaging of the interactions between all components and sequential monitoring of assembly, and its intermediate stages, as well as the stages of in vitro mitotic breakdown [18].

Further Access to Nuclear Envelope Structure

Once isolated and attached to an adhesive substrate, the amphibian (or insect) nuclear envelope is available for further molecular dissection, which we have pursued in 3 ways, proteolytic or detergent extraction (or a combination of both) and various mechanical manipulations which also expose fresh structure [17, 19].

Proteolysis

Isolated NEs from *Xenopus* or *Triturus* were incubated for 5 mins in concentrations of 1, 10 or 100 µg/ml trypsin (Worthington Biochem Corp, Freehold NJ, USA) in 5-1 buffer at room temperature. Membrane structure of the NE was unaffected, but the proteins of the NPC were dismantled in a sequential and progressive manner. With 30 million pores on each NE, it is a relatively simple matter to achieve suitably representative morphology at different concentrations. The cytoplasmic face of the NPC showed sequential removal of cytoplasmic granules, leading to exposure of the surface of the cytoplasmic coaxial ring, followed by breakdown of the ring itself, starting from a single point and proceeding circumferentially from that point, leading to 'horseshoe' and then C shaped remnants (Fig. 6). On no occasion have we observed more than a single break in the cytoplasmic coaxial ring, indicating the existence of an initial trypsin sensitive site followed by successive removal of building blocks from that point circumferentially, with the whole disassembly possibly indicative of the 'physiological' breakdown stages in normal mitotic disassembly of NPCs. At the same time, internal elements of the NPC become successively exposed, and the overall breakdown continues until only NPC sized holes are visible in a largely 'depopulated' cytoplasmic face of the NE. On the nucleoplasmic side, similar changes are mirrored in the breakdown of NPC baskets and the nucleoplasmic coaxial ring, although where remnants of the nuclear envelope lattice are retained, it appears to be more trypsin resistant than the NPCs [19].

Detergent extraction

Incubation of unfixed isolated NEs for 5 minutes in 0.5% Triton X 100 (Sigma Chemicals, St. Louis, USA) in 5 : 1 buffer produces a 'converse' of proteolytic incubation, in that the NPCs are unaffected, but the membranes of the NE are removed, exposing the underlying filamentous network of the nuclear lamina.
Figure 6: Cytoplasmic view of individual nuclear pore complexes after incubation with increasing concentrations of trypsin (a= 10 µg/ml, b= 100 µg/ml) for 5 mins. The cytoplasmic ring clearly exposed by removal of surface granules is a, shows a regular 8 sub-unit structure (arrowheads) and the side elevation of the surface profile is also apparent (arrows). In B, the coaxial ring is largely removed, with large holes appearing in the centre of the disassembling pore complexes. (Bar= 100 nm).

This is apparent from both cytoplasmic and nucleoplasmic sides of the NE, although as around two-thirds of NPC structure sits ‘above’ the lamin network, less details of lamin organisation and interaction with NPC structure is apparent from the cytoplasmic side. Occasionally, probably due to mechanical disruption, NPCs become partially detached and are seen in ‘side elevation’ lying on the surface of the lamin network. Viewed from the nucleoplasmic side, the interaction of the NPCs with the lamin network is clearly visualised. Although many individual lamin filaments have become detached and disrupted, the remainder appear to insert into the base of the nucleoplasmic coaxial ring, often in intermediate positions between the attachments of the fibres of the NPC basket structure (Fig. 7).

Combined proteolysis and detergent extraction

Use of trypsin and detergent in combination allows further access to the ‘core’ structures of the NPC, as they may be ‘hidden’ by a combination of both surface proteinaceous material and NE membranes. Visualisation of NPCs after both brief detergent and trypsinisation has clearly indicated the radial arm structure at the periphery of the NPC core, exposed by removal of both peripheral proteins (the coaxial rings) and the NE membranes, particularly the pore membrane, [19].

Fracturing techniques for nuclear envelopes

‘Non-biochemical’ access to internal NPC structure has been achieved by two simple, apparently crude, but undoubtedly effective ways of separating subunit NPC structure. If freshly isolated unfixed nuclear envelopes are ‘rolled’ over the surface of the Si chip during isolation, the NPCs separate in the plane of the membrane, leaving their cytoplasmic coaxial rings attached to the surface of the chip, and exposing underlying struc-
Figure 7: Unextracted (7A) and Extracted (7B) views of the nucleoplasmic face of *Xenopus* oocyte nuclear envelope. In 7A, lamin filaments can be seen in relief (arrowed) at the surface of the inner nuclear envelope membrane. In 7B, after Triton 100 X removal of this membrane, the lamin filaments are apparent, attached to the nucleoplasmic coaxial ring of the NPCs. Mechanical stress has resulted in some disorganisation of the lamin organisation overall, which appears to have an orthogonal arrangement in undisturbed NE membranes. (Bar= 100 nm).

The Visualisation of Labelled Molecules by FEISEM

As well as exposing structural details of the NE and NPC, we have also attempted to characterise these observations by using antibodies or lectins to identify specific nucleoporins (nuclear pore proteins). Although there are probably 100 different proteins contributing to the NPC structure, only a few have been characterised in situ using immuno-TEM, and this has often been in cryosections, which further inhibit exact localisation to specific pore substructures due to their relatively limited contrast [30]. Whereas vertical sections through the NPC show profiles, and FEISEM could be considered limited to surface imaging, FEISEM preparations will routinely expose enormous numbers of both cytoplasmic and nucleoplasmic faces of the NPC, as well as indicating...
Figure 8: View of isolated NE from Xenopus oocyte that has been ‘rolled’ over the surface of the Si chip during isolation, leaving cytoplasmic components of the NPCs attached to the surface of the chip (foreground). (Bar=200 nm).

The sites of direct binding to the individual structures of the NPC, and adjacent structures. As many of the nucleoporins are characterised by the presence of n-acetyl-glucosamine, and active transport through the pore is blocked by wheat germ agglutinin (WGA) [42], (which binds to n-acetyl-glucosamine), we have also been interested to pinpoint as precisely as possible the sites of binding of WGA.

Clearly, whole primary antibody molecules which are 15 nm in length, with secondary antibody molecules bound to them will potentially have a “radius of uncertainty” of several nanometres, with respect to the precise site of binding of the primary antibody to the antigen. In this situation, spatial resolution of around 2 nm may not be the limiting factor in pinpointing the molecular site of antibody binding. Our own approach has been initially to use conventional primary antibody binding, followed by secondary antibodies bound to colloidal gold, usually of 5 or 10 nm diameter, as well as WGA directly complexed to 5 or 10 nm gold. We have visualised the colloidal gold using a retractable solid state backscatter detector (KE Developments, Cambridge, UK). In order to retain the maximum amount of structural detail in the same image as the gold localisation, we have coated the gold labelled specimens with approximately 2 nm of Chromium, so that the optimal secondary electron signal from the specimen is retained. Because of the relative atomic Z numbers (Cr, 24; Au, 79) there is little interference from the backscatter electron (BSE) signal generated by the Cr coating compared with BSE signal from the colloidal gold. In fact, there is more BSE signal from areas of increased osmiophilic fixation (Os, Z=76), but as this is diffuse rather than particulate, it is not usually a problem with respect to gold localisation. Indeed, in NE preparations, the dense concentrations of...
osmiophilic material in the NPC such as the coaxial rings, are clearly observed by BSE imaging, although with significantly less topographical detail than secondary electron (SE) imaging (Figs. 10, 12abc). In order to optimise the visualisation of the gold colloid relative to the SE image of the structure to which it is bound, we have reversed the BSE signal, and used a standard mixing box to (ETPRA, Australia) superimpose it in the SE signal. In this way the gold colloidal appears as typical 'black dots' familiar from TEM gold labelling, superimposed on the typical SE visualisation of 3D surface topography (Figs. 11, 12). This type of visualisation will also often demonstrate the protein 'shell' which coats the gold colloid, not usually observed in TEM (Figs. 12de). To date, we have only been successful with gold down to 5 nm in diameter with this approach, due to limitations in our system for optimising and mixing BSE and SE signal levels, but we feel that this could be improved to smaller gold colloid, if not down to 1.4 nm nanogold (Nanoprobes, Stony Brook, NY, USA), at least to nanogold which has been silver enhanced to around 3 nm in diameter. These smaller gold probes have the advantage of direct chemical bonding to the molecule of interest, rather than depending on a less stable electrostatic interaction between colloid and protein, but are best used with Fab fragments rather than whole antibodies, again reducing 'the radius of uncertainty'.

The Visualisation of NPC Transport by FEISEM Nuclear Import

For many years, nuclear import has been visualised by microinjection of colloidal gold carrying a necessary nuclear localisation sequence (NLS), mainly in the excellent studies of Feldherr et al. [12, 13]. Thin section TEM studies show binding of gold colloid, up to a maximum diameter of 25 nm, first to the periphery of

Figure 10: (A) SE image of cytoplasmic face of *Xenopus* labelled NE with antibody to P180 (a gift of Dr U Aebi). (B) BSE image of same area. The colloidal gold is apparent as a clearly discrete signal, whereas the increased BSE signal from the osmiophilia of the pore complex is more diffuse. (Bar=100 nm).
Figure 11: Mixed image of reversed contrast BSE signal mixed (about 60:40) with SE signal during signal detection, guaranteeing that registration between the two images is retained. WGA - gold labelling of cytoplasmic face of NPCs on *Xenopus* NE, 10 nm diameter gold. (Bar = 100 nm).

the NPC at the tips of the cytoplasmic filaments, followed by movement down these filaments, across the surface of the NPC, and movement through the central core (Fig. 12i). As TEM does not readily visualise NPC basket structure, the route through this inner aspect of the pore complex remains uncharted. In our own studies, we have microinjected 10-15 nm colloidal gold, complexed with nucleoplasm, which has 4 NLS (nuclear location sequences) per molecule. We have cut sections at a thickness which is equivalent to the diameter of the pore (100-120 nm) in an attempt to generate whole profiles of NPCs somewhere in the section. Making stereopairs in the TEM has helped with 3D reconstruction of the passage of gold through the pore (Allen, unpublished). We have also isolated NEs from microinjected nuclei, and observed gold binding to the surface of the cytoplasmic filaments and migration to the centre of the pore. Although passage through the NPC core is ‘single file’ 15 or more nucleoplasm gold particles can be bound at once to the cytoplasmic filaments. Passage through to the nucleoplasmic side, again using SEM stereopairs appears to result in emergence through the centre of the NPC basket ring (Fig. 12fgh).

**Nuclear pore export**

The molecular requirements for nuclear export are less well characterised than those for import, although mRNA complexed gold will be exported after microinjection [10, 29]. We have investigated a system which allows direct visualisation of nuclear export in physiologically normal circumstances. Polytene nuclei in insect salivary glands contain giant (polytene) chromosomes which generate enormous numbers of mRNA transcripts for the production of salivary proteins. These transcripts are liberated from permanently amplified regions of the polytene chromosome (Balbiani Rings (BR)) in the form of RNA and ribonucleic protein (RNP) granules 50 nm in diameter. These Balbiani ring granules attach to the NPC baskets, where they appear to ‘dock’ in the correct orientation, before becoming ‘unravelled’ to allow passage through the NPC with the RNP fibre exiting the pore rather like toothpaste out of a tube. We have visualised both the binding to the NPC basket, and export directly from surface imaging, and using deresin methods (see above) have observed BR granule binding to the basket in ‘side elevation’. Our findings are in agreement with those of Mehlin *et al.* [25] who showed unravelling of the BR granule by TEM tomography, but did not demonstrate the involvement of the NPC basket, as this is not normally apparent in TEM thin sections.

**Conclusions**

Field emission in lens scanning EM (FEISEM) offers the possibility of equivalent effective resolution to
Figure 12: FEISEM of microinjected 5 nm nucleoplasmic gold, visualised by a) secondary electron (SE) imaging; b) Backscatter electron (BSE) imaging; and c) electronically mixed imaging of a and b. Mixing of unreversed contrast BSE imaging with SE imaging is not optimal with respect to exact localisation of the colloidal gold. de: SE and BSE/SE mixed images of microinjected 10 nm nucleoplasmin gold in a central position on the cytoplasmic face of the NPC. Topography is insufficient for gold colloid characterisation, as similar sized spheres, (arrowed) are clearly not gold in the mixed signal, in which the contrast reversed BSE image from the colloidal gold is more readily apparent than A-C. fgh: Microinjected nucleoplasmin 10 nm gold colloid in passage through the NPC. 12f shows the cytoplasmic face of the NE, 12g and h, the nucleoplasmic face, with gold ‘exit’ through the inner basket ring of the NPC basket (arrowed). i (on page 160, top right column): Conventional thin section TEM showing NPC transport of Nucleoplasmin gold during passage through the central region of the NPC. (Bar (all cases)= 100 nm).

conventional TEM for biological specimens, with the advantages of bulk specimens and surface imaging, which allows direct 3D visualisation of interfaces within the cell such as the nuclear envelope. Without the constraints of thin sections or single layers of molecules, surface imaging can be applied to any aspect of the cytoplasm or nucleus that can be accessed, either as a result of isolation, proteolytic or detergent exposure, de-resining of sectioned material, or simple fracturing techniques not requiring the high tech approach associated with cryofracturing. It also offers the possibility of antibody characterisation using colloidal gold labelling,
and also direct observation of nucleo-cytoplasmic transport in situ within the cell, viewed from either the nuclear interior or the cytoplasmic face of the NE. Thus, FEISEM can provide useful complementary information to extend our understanding of the 3D organisation of nuclear surfaces and interfaces.

References


Discussion with Reviewers

M. Malecki: How would you modify protocols for isolation of nuclei from cells cultured in suspension versus on substrates?

Authors: For nuclear isolation, the cells could be removed from the substratum (either scraping or trypsin) and then homogenised after swelling in much the same way. Because of the high probability however of the attachment to the substratum generating polarising influences on the cell and its nucleus, I think the approach illustrated in Fig. 3, where the nucleus and its contents are exposed in situ is potentially a valuable approach. Cells in suspension may well be polarised in some way, but obviously there is no method of either ascertaining, or maintaining such polarity. Attached tissue culture cells are known to require a certain amount of spreading over the substratum before crucial processes such as DNA replication can be achieved.

M. Malecki: How does spinning down of isolated nuclei on the poly-I-lysine (PLL) coated silicon chips affect nuclear architecture? What is the adhesion rate?

Authors: As the nuclei are usually fixed in suspension prior to attachment to PLL coated silicon chips, we do not envisage any alteration as a result of nuclear attachment. The nuclei show no distortion after attachment, retaining their spherical morphology. Usually a brief spin (5 min, 1000 g) is adequate for a high yield of nuclear attachment.

M. Malecki: Did you attempt to reduce the radius of uncertainty by conjugating gold beads to primary FABs?

Authors: We have not attempted this yet, but fully agree that small gold (1-3 nm) directly conjugated to FAB fragments of primary antibodies should considerably improve the resolution of labelling. The resolving power of FEISEM is capable of this, although the immunology could be demanding.

M. Malecki: Would you be willing to share your observations on nuclear pore architecture prepared by: 1) isolation in buffers followed by fixation: 2) fixed in situ followed by their exposure: 3) rapid freezing followed by their exposure?

Authors: We can respond to parts 1 and 2, we have not tried 3 yet. Isolation in buffers followed by fixation is
our standard approach to NPC structure by FEISEM (see refs. 13-18 and 43). 'Fixed in situ followed by exposure'—this is a difficult approach, as the fixation will preclude the separation of structures such as the nuclear envelope from their surrounding material, namely cytoplasm and nucleoplasm. We have however isolated nuclei in the presence of glutaraldehyde, and noticed a significant increase in the length of cytoplasmic filaments retained on the cytoplasmic face of the nuclear pore complex (NPC). It is important to bear of this type of approach in mind when considering methods which will allow visualisation of organelles such as NPCs in interaction with elements of both the nucleus and cytoplasm.

Additional Reference