Scanning Microscopy

Volume 1 | Number 1 Article 28

11-30-1986

Colloidal Gold - A Powerful Tool in Scanning Electron Microscope Immunocytochemistry: An Overview of Bioapplications

G. M. Hodges
Lincoln's Inn Fields

J. Southgate
Lincoln's Inn Fields

E. C. Toulson
Jeol (UK) Ltd.

Follow this and additional works at: https://digitalcommons.usu.edu/microscopy

Part of the Life Sciences Commons

Recommended Citation

Hodges, G. M.; Southgate, J.; and Toulson, E. C. (1986) "Colloidal Gold - A Powerful Tool in Scanning Electron Microscope Immunocytochemistry: An Overview of Bioapplications," *Scanning Microscopy*: Vol. 1 : No. 1 , Article 28.
Available at: https://digitalcommons.usu.edu/microscopy/vol1/iss1/28

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Scanning Microscopy by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.

Scanning Microscopy, Vol. 1, No. 1, 1987 (Pages 301-318) 0981-7035/87$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

COLLOIDAL GOLD - A POWERFUL TOOL IN SCANNING ELECTRON MICROSCOPE IMMUNOCYTOCHEMISTRY: AN OVERVIEW OF BIOAPPLICATIONS

G M Hodges*[1], J Southgate[1], E C Toulson[2]

Tissue Interaction Laboratory, Imperial Cancer Research Fund,
Lincoln's Fields, London WC2A 3PX, UK[1];
Jeol (UK) Ltd., London NW9 0JN, UK[2].

(Received for publication August 03, 1986, and in revised form November 30, 1986)

Abstract

Colloidal gold may be conjugated to a wide variety of macromolecules, provides a versatile system for immunocytochemical studies by various types of microscopy (light and fluorescent microscopy, scanning (SEM) and transmission (TEM) electron microscopy), and is significantly contributing to the development of SEM immunocytochemistry as a routine analytical procedure.

A comprehensive overview has been compiled of the literature on SEM bioapplications of colloidal gold. This is illustrated through a selected series of studies focussing on a) cell surface receptor-ligand interactions; b) expression of cell surface lectin-binding sites; c) surface distribution of extracellular matrix components; and d) visualization of gold-labelled cytoskeletal elements with emphasis on the use of backscattered electron imaging as a powerful analytical adjunct in the development of SEM immunocytochemistry.

Key Words: Colloidal gold; scanning electron microscope immunocytochemistry; backscattered electron imaging; biomedical applications; cell surface labelling; extracellular matrix labelling; detergent extraction.

*Address for Correspondence:
G M Hodges,
Tissue Interaction Laboratory,
Imperial Cancer Research Fund,
Lincoln's Inn Fields,
London, WC2A 3PX
Phone No: 01-242-0200

Introduction

Scanning electron microscopy (SEM) has had a relatively limited penetration into the area of immunocytochemistry. Yet, the ability to couple the technology of immunocytochemistry to the SEM offers a tremendous potential for expanding the repertoire of useful information that may be obtained from immuno- and nonimmuno-labelling procedures.

The microscopic analysis of specific molecular units on the surface membranes of cells tagged with markers visible in scanning EM was first introduced by LoBuglio et al (1972). Since then, a variety of particulate, enzymatic and emissive markers have been explored at SEM level as visualization tags for target molecules with the advantages and disadvantages of such markers extensively discussed in a series of reviews (Nemanic 1975; Molday 1977,1983; Brown and Revel 1978; de Petris 1978; Hoyer et al 1979; Goodman et al 1980; Molday and Maher 1980; Horisberger 1981; Hoyer and Bucana 1982; Polliack and Gamliel 1983; Hicks and Molday 1984). Despite the potential of SEM immunocytochemistry highlighted in these publications, there has remained, as previously discussed (Hodges et al 1984), a putative appreciation of this potential. In large part, the reticence in applying the technique of immunocytochemistry for use in the SEM has reflected limitations in SEM-orientated marker, instrumentation and specimen preparation technologies. However, recent developments in immunocytochemical marker systems - and notably the introduction of the colloidal gold marker system; in SEM instrumentation - and notably the improvement in sensitivity of backscattered electron detector systems; and, in SEM biological specimen preparation options - and notably that of techniques aimed at exposing specific internal constituents of cells and tissues offer significant improvements to the practice of SEM immunocytochemistry.

This paper is designed to briefly review those developments and to focus on studies that demonstrate the effective contribution of the colloidal gold marker system in cell labelling investigations coupled to SEM analysis procedures.

Marker systems for SEM

An ideal marker for SEM should meet a number of basic prerequisites detailed discussions of which have been given in recent reviews (Molday 1977, 1983; Brown and Revel 1978; Molday and Maher 1980; Polliack and

Gamliel 1983; Hicks and Molday 1984; Hodges et al 1984). In brief, markers should be selected on the basis of certain distinct properties with respect to size and shape, stability, capability of interaction and of strong binding with a range of ligand molecules, and minimal natural binding affinity for biological surfaces. Furthermore, the relative ease of manufacturing marker and marker-protein complexes, the relative stability and bioactivity of marker-protein complexes, and the possibility of visualizing markers by different modes of microscopy and by different analytical techniques within each mode of microscopy are yet further factors which can influence the choice of particular marker systems.

Of the variety of markers developed for electron microscopy and explored for use in the SEM it is the colloidal gold marker system first introduced for this mode of microscopy by Horisberger et al in 1975 which has, to paraphrase Beesley (1985), emerged as the new revolution in SEM immunocytochemistry. By virtue of its properties (Table 1) colloidal gold has provided the opportunity for establishing scanning EM as an effective microscopic approach for the detection of target molecules (Horisberger 1979, 1981, 1985; Goodman et al 1979, 1980; Hicks and Molday 1984; Hodges et al 1984; see section below on SEM bioapplications of colloidal gold). Under the SEM gold particles produce a high emission of electrons which can be detected by either secondary (SEI) or backscattered (BEI) electron imaging, while X-ray microanalysis will detect the characteristic X-ray signals emitted by gold. Because of these optimal properties for detection by electron microscopy, gold particles can be readily distinguished and provide for accurate macromolecular mapping of biological surfaces by SEM (Hoyer et al 1979; Horisberger 1981, 1985, Walther et al 1983, 1984; Walther and Müller 1985, 1986) de Harven et al 1984; Hicks and Molday 1984; Hodges et al 1984; Nava et al 1984).

The general conditions under which colloidal gold can be formed, the general considerations about adsorption of biological macromolecules to colloidal gold, the development of gold-dextran particles as an alternative gold marker, and detailed protocols relevant to the manufacture both of gold markers and of gold probes are discussed in several comprehensive reviews which emphasize different aspects of these subjects for example:- Goodman et al (1979, 1980, 1981); Horisberger (1981, 1985); Roth (1982, 1983); de Mey (1983); Handley and Chien (1983); Hicks and Molday (1984); Hodges et al (1984); Beesley (1985); Lucocq and Roth (1985).

Instrumentation

Typically, SEM imaging of cell surface-bound gold markers has been by secondary electron imaging (SEI) although this is only true for larger gold particles in the size range of 30-40nm (cf. Horisberger 1981; Albrecht et al 1986). The high emission of secondary electrons from the gold serves to identify the gold particles as bright, well-defined objects on cells which have been coated either with a heavy metal or with carbon prior to examination by SEM (Horisberger 1981; Hodges et al 1984) (cf. Figs 10;13). This approach has proven extremely useful in investigative studies as given in several review articles (Goodman et al 1980; Horisberger 1981, 1985; Hodges et al 1984) but nevertheless, can be the subject of criticism with two principal limitations being identified (Goodman et al 1980; Horisberger 1981; Hodges et al 1984; Nava et al 1984).

A first constraint is that, conventionally, metal coatings have been used to allow the generation of sufficient signal for good resolution of surface features. However, this has the disadvantage of generally introducing a 10 to 20nm mask with the inherent potential of obscuring both small surface structures and small gold particles; of altering size and shape of such structures and markers; and of generating metal decoration on surface features thereby risking erroneous interpretations (Echlin 1981; Horisberger 1981; Peters 1984, 1985). Non-coating techniques are available to render biological specimens conductive (Murphy 1978, 1980) thereby obviating the requirement for metal film deposition. Nevertheless topographic resolution may be constrained, but the development of high-resolution ultra-thin metal or carbon coatings could overcome these limitations (Peters 1984, 1985; Walther et al 1984; Soligo et al 1986).

SEI visualization of gold markers imposes a second constraint in that the complex topography of many cell surfaces may mask small or sparse markers; furthermore, small surface membrane projections, budding viruses, or various contaminating protein aggregates, cellular debris or particles of unknown origin can be of marker size and shape, and prove difficult to distinguish from single or clumps of gold particles.

Backscattered electron imaging (BEI) in the scanning EM has been used extensively for the localization of medium to high atomic number elements in low atomic number biological matrices (Becker and Sogard 1979; Becker and Geoffroy 1981). Recent improvements in sensitivity of BEI imaging detectors now indicate, by virtue of the high electron backscattering coefficient of gold, that BEI would provide a valuable alternative to SEI in the SEM imaging of gold-labelled specimens. Such BEI imaging detectors include the single crystal scintillators of the YAG (ytrium-aluminium garnet) -type as used by Walther et al (1983, 1984; Walther and Müller 1986) and Autrata et al (1986) or the improved solid state detectors as discussed by Reimer et al (1979), Oatley (1981) Reimer (1984). Therefore, in that BEI provides atomic number/density contrast (Becker and Sogard 1979), the atomic number contrast of gold particles (Z=79) is well recognized when the surface of carbon- or non-coated cells is observed by BEI (Trejdosiewicz et al 1981; Hodges et al 1984, Walther et al 1983, 1984; Walther and Müller 1985, 1986; de Harven et al 1984; Nava et al 1984; Horisberger 1981, 1985; Soligo et al 1986). Use of BEI allows gold particles to be seen with good contrast on the surface of specimens (cf. Figs 7;14); and within or beneath the specimen surface through application of appropriate intracellular immunolabelling techniques (cf. Fig 23) though physical parameters limit the depth of visualization (Becker and Sogard 1979; Peters 1985). That BEI imaging of specific intracellular deposition of various elements (principally of high atomic number) may be directly correlated, at the level of the individual cell, with such parameters as the surface morphology of the cell has been clearly demonstrated in SEM enzyme cytochemistry studies (Soligo et al 1981; 1986). The unambiguous detection of gold particles by BEI also clearly identifies contaminating micro-debris (unless this contains high atomic number elements thereby generating a BEI signal of comparable intensity to that given by gold particles). Furthermore, BEI offers the potential for applying computer-aided on-line image analysis techniques to gold-labelling studies and developing SEM immunocyto-

Table 1

ADVANTAGES OF COLLOIDAL GOLD AS A MARKER IN EM IMMUNOCYTOCHEMISTRY

- monodisperse gold sols with gold particles of uniform size and shape can be rapidly, reproducibly and inexpensively prepared in a particle size range of 2-150nm mean diameter. Gold sols remain stable for many months under appropriate storage conditions at 4°C.
- the particulate nature of colloidal gold allows fine localization of marked sites; the size range guarantees high flexibility in lateral resolution.
- gold particles are negatively charged and can be complexed by non-covalent electrostatic adsorption with various macromolecules (e.g., staphyloccocal protein A, immunoglobulins, lectins, toxins, glycoproteins, enzymes, streptavidin, hormones, peptide antigens conjugated to bovine serum albumen) forming stable and bioactive gold-ligand complexes termed gold probes. Under appropriate storage conditions these will remain stable and retain much of their bioactivity for many months at 4°C: high labelling flexibility is guaranteed through a wide choice of reagents.
- gold particles demonstrate high electron density because of the high atomic number of gold and are capable of strong emission of secondary and backscattered electrons: these physical characteristics make gold particles excellent markers for TEM and SEM.
- the high electron backscattering coefficient of gold suggests that the enhanced contrast of backscattered electron imaging could provide a superior alternative for visual or computer-aided quantitative analysis of target molecules; while
- the characteristic X-ray signals emitted by gold could be used to image and quantify cell-bound gold markers by application of X-ray micro-analytical techniques and appropriate computer programmes.
- double- or multiple-labelling of different target sites is possible by the application of monodisperse gold probes of various sizes.
- quantification can be achieved by direct counting of gold particles.
- due to the low degree of non-specific adsorption of gold probes to speciment surfaces the signal-to-noise ratio is very high.
- because of high binding constants of gold probes to specimen surfaces, biological samples can be processed with minimal loss of gold particles.
- because gold particles absorb or reflect light and can be amplified by silver enhancement procedures they are applicable for a variety of light microscope marking techniques as well as non-microscopical procedures (including immunoblotting and immunoprecipitation) and thereby provide a range of correlative methodologies.

chemistry into a quantitative biochemical tool. Yet another facility is the combination of the BEI signal with the SEI signal which has the considerable advantage of providing for a direct correlation between the distribution of labelled sites and the topography of the cell surface (de Harven et al 1984; Soligo et al 1986).

Specimen preparation

In broad terms, scanning EM has been used mostly to examine the topography of naturally exposed surfaces of cells and tissues. Yet, in many studies it is essential to explore both the surface features and spatial relationships of cells located within tissues, and to visualize intracellular structures of interest. Furthermore, if SEM is to become a useful tool in immunocytochemistry, development of specimen preparation procedures are essential which allow localization of target molecules anywhere in cells and tissues with a high degree of sensitivity. As a consequence, more recent efforts have been directed toward developing preparative methods for biological materials effective in exposing specific internal constituents of tissues and organs thereby extending the morphological and analytical roles of the SEM: several reviews provide discussion of different aspects of available preparative methodologies and their advantages and disadvantages (Boyde and Wood 1969; Hollenberg and Erickson 1973; Boyde 1975; Waterman 1980; Bell 1981; Tanaka 1981; Bell and Stark-Vancs 1983; Tanaka and Mitsushima 1984; Borwein 1985; Carr et al 1987).

Review of the immunolabelling literature indicates that sample preparation can take one of many forms (Table 2) and that the manner in which the specimen is presented for immunolabelling determines the type of information that can be obtained from the sample. Much of the SEM immunocytochemical work to date has relied upon non-embedding techniques applied largely to investigations of cell surface antigens and lectins (Goodman et al 1980; Horisberger 1981, 1985; Hicks and Molday 1984; Hodges et al 1984). This literature has clearly delineated the potential advantage offered by immunocytochemistry applied to SEM in that the molecular organization of entire upper surfaces of cells or tissues is amenable to examination. By contrast is the restricted analysis offered by small segments of limited surface areas available for viewing in a cell profile in a thin section. Yet, as discussed earlier, preparative procedures aimed at intracellular analysis are clearly available such that SEM immunocytochemistry need not be restricted to the examination of cell surfaces, but rather should be able to permit the overall and integral distribution of target molecules to be established.

Labelling procedures

Irrespective of the mode of microscopy, it is well established that immunocytochemistry follows a basic

Table 2

OUTLINE OF PREPARATIVE TECHNIQUES EMPLOYED TO DATE IN COLLOIDAL GOLD IMMUNOCYTOCHEMISTRY

	Technique	LM[1]	TEM[2]	SEM[3]
a)	Non-embedding techniques			
-	(Fix) - label - view Includes use of: vibratome sections; tissue slices; cells in suspension or in monolayers; whole mount preparations; cell permeabilization and/or extraction techniques; detergents and immunoglobulin fractions to increase tissue penetration; fracture-label.	+	+	+
b)	Pre-embedding techniques			
-	(Fix) - slice - label - embed - section - view Includes use of: vibratome sections; tissue slices; freeze-fracture blocks; detergents and immunoglobulin fractions to increase tissue penetration.	+	+	+
c)	Post-embedding techniques			
-	(Fix) - embed - section - label - view Includes use of: paraffin (4-10μm) sections; polyethylene glycol (0.2-0.5μm) sections; semithin resin (0.2-1μm sections); ultrathin resin sections; semithin (0.5-1μm) or ultra-thin (600-100μm) cryosections.	+	+	-
d)	Immunonegative stain technique			
-	(Fix) - (cryosection) - label - negative stain - view	-	+	-
e)	Immunoreplica technique			
-	(Fix) - label - critical point dry - shadow - view	-	+	-
f)	In-situ hybridization	+	+	-

1, 2 For reviews of preparative techniques and fixation conditions used in LM and TEM applications see Bullock and Petrusz 1982, 1983, 1985; de Mey 1983; Polak and Varndell 1984; van den Pol 1984; Wolosewick 1984; Beesley 1985; Sternberger 1986.

3 For review of preparative techniques and fixation conditions used in SEM applications see Goodman et al 1980; Horisberger 1981, 1985; de Mey 1983; Hicks and Molday 1984; Hodges et al 1984.

strategy with cytochemical marking providing for the visualization of target molecules. Such targets are identified and localized by high-affinity specific binding with identifier molecules including antibodies (for antigens), lectins (for polysaccharides and glycoproteins), enzymes (for their substrate, e.g., polynucleotides), ligands (for their receptor or binding site), and derivatized polynucleotides. In turn, identifier molecules are marked by application of visualizers appropriate to the intended mode of analysis. Such visualizers (or markers) may interact either directly with the identifier, or indirectly through linking molecules that bind to the identifier: linkers for antigen-bound antibodies include secondary antibodies, protein A, or antigens. Lastly, visualizers are then observed using appropriate modes of observation.

As a consequence, cell labelling procedures for SEM immunocytochemistry do not differ in principle from those used for light microscope or TEM immunocytochemistry and can be broadly grouped into direct, indirect, or sandwich techniques. The methods available for labelling more than one target molecule on a sample are, again, similar in basic principle. Different aspects of this labelling technology are comprehensively discussed in a series of publications a number of which include the presentation of detailed protocols (de Petris 1978; Sternberger 1986; Bullock and Petrusz 1982, 1983, 1985; Osborn and Weber 1982; Roth 1982, 1983; Polak and Varndell 1984). Several sources also provide extensive background data on the specific subject of labelling procedures for SEM immunocytchemistry (Molday 1977, 1983; Brown and Revel 1978; Molday and

Maher 1980; Horisberger 1981, 1985; Hoyer and Bucana 1982; Hicks and Molday 1984; Hodges et al 1984). Also discussed in these two series of references are the essential conditions and constraints influencing immunocytochemical localization of target molecules with significant considerations including the adequate preservation of antigenicity and tissue structure, efficiency of labelling, sensitivity of technique, and specificity of ligands. The question of absolute quantification of cell-bound markers is also raised in that many variables including target accessibility, target density, size of marker complex, number of immunoglobulins per marker, and steric hinderance may all influence binding and therefore quantification.

One advantage of colloidal gold as a marker is its particulate nature and several methods including direct counting, spectrophotometry, radioassay and X-ray analysis have been proposed to quantify gold particles bound to biological samples, although caution has to be exercised in the interpretation of numbers of gold particles to numbers of target molecules as indicated above. This problem is possibly more acute in SEM immunocytochemistry where present-day instrumental resolving capabilities (3-10nm) coupled with surface conductive coating requirements (5-20nm thickness) (but see Peters 1985) imposes in general, the need for markers of a relatively large size (see Table 3). This clearly renders more difficult the establishment of a good stoichiometry between marker and target site (Horisberger 1981, 1985). As a consequence, the precision with which most target molecules can be localized by SEM has been severely limited in that the larger-size markers used in many present-day SEM labelling studies will sterically preclude a one-to-one correspondence of marker to target molecule depending on the density and distribution of the binding sites (cf. Walther and Müller 1985). Furthermore, as a result of steric hinderance, the number of bound gold particles has been shown to decrease, sometimes abruptly, when the particle size is increased. However, the detection of smaller (5-15nm) gold particles using backscattered electrons and higher-resolution instrumentation (Walther et al 1984, Walther and Müller 1985, 1986) will allow for more precise localization of target molecules by SEM. Several sources provide further comprehensive discussions on labelling efficiency and precision of quantification (Brown and Revel 1978; de Petris 1978; Horisberger 1979, 1981, 1985; Hoyer et al 1979; Sternberger 1986; Kraehenbuhl et al 1980; Molday 1983; Molday and Maher 1980; Bullock and Petrusz 1982, 1983, 1985; Hicks and Molday 1984; Hodges et al 1984).

SEM bioapplications of colloidal gold

Gold labelling of target molecules has been applied to a range of problems many involving TEM, and to an increasing extent light microscopy although, by comparison, relatively few yet involve SEM. In TEM, the gold marker system has been extensively used to study cell surface antigens, receptors or glycoconjugates; receptor-mediated or fluid-phase endocytosis and cell surface redistribution phenomena; intracellular antigens, lectin-binding sites, receptors and nucleic acids; in situ hybridization; membrane lectin-mediated cellular glycoprotein uptake; synthesis and secretion of proteins and polypeptides; intracellular topology of glycosylation; glycocalyx domain formation; vascular permeability; and localization of extracellular matrix and basal lamina components (for general reviews see Goodman et al 1980, Horisberger 1981; de Mey 1983; Handley and Chien 1983; Roth 1983; Beesley 1985). The versatility and usefulness of colloidal gold as a marker is emphasized by this broad spectrum of applications covering areas of biological and biomedical study related to receptors, endocytosis, transcellular pathways, enzyme-substrate reactions and functions of proteins.

In SEM, there exists a more limited spectrum of published applications of the gold marker system (Table 3). These have focussed, almost exclusively, on the study of cell surface antigens and glycoconjugates with good high-resolution imaging of gold particles by BEI scanning EM being reported in several studies (Trejdosiewicz et al, 1981; de Harven et al 1984; Hodges et al 1984; Nava et al 1984; Walther et al 1984; Soligo et al 1986; Studer and Hermann 1986; Walther and Müller 1986). Simultaneous detection of multivarious target molecules has been also explored in SEM studies either using gold particles of different sizes (Horisberger et al 1975; Horisberger and Rosset 1977b) or double-labelling with gold particles and ferritin (Hoyer et al 1979).

Below we present a sampling, from our own experience, of SEM bioapplications of colloidal gold focussing on recent studies related to a) cell surface receptor-ligand interactions; b) expression of cell surface lectin-binding sites; c) surface distribution of extracellular matrix components; and d) visualisation of cytoskeletal elements. The preparation of the various gold-ligand complexes and the labelling of the different specimens were carried out using procedures as previously described by Goodman et al (1981) and by Hodges et al (1982, 1984).

Localization of cell surface antigens (I)

Expression and surface distribution of a urothelial membrane-associated antigen (UMA) (Hodges et al 1982; Hodges and Kenemans 1982; Trejdosiewicz et al 1984).

Procedure for gold-labelling of tissues. The bladders of young female Wistar rats (130g) given a single non-tumorigenic intravesicular dose of 1.5mg N-methyl-N-nitrosurea (MNU) (in order to elicit benign urothelial hyperplasia and a regenerative process of normal urothelial differentiation) were excised, placed in Hanks' solution, dissected into quadrants, pinned out on dental wax, and prefixed with freshly-prepared 4% paraformaldehyde contained in 0.1M Sorensen's phosphate buffer pH7.4 for 30 min at ambient temperature. All subsequent reactions were performed at ambient temperature and each step was followed by at least three washes for 5-10 min in TBS (0.15M NaCl, 10mM Tris-HCl, pH7.4). The tissues were incubated with the monospecific affinity-purified UMA antibody (Trejdosiewicz et al 1984) (diluted 1:4 in TBS) for 60 min; washed; then covered with gold-labelled secondary antibody (undiluted goat anti-rabbit-gold - 45nm dia probe) for a further 60 min. Specificity controls included the omission of first antibody, or the use of irrelevant first antibody (anti-fibronectin).

At the end of the incubation period, the tissues were washed; postfixed with 2.5% glutaraldehyde in 0.1M Sorensen's phosphate buffer pH7.4 for 24h at ambient temperature; processed through an osmium -thiosemicarbazide schedule; dehydrated through graded ethanols; critical point dried from liquid carbon dioxide; and coated with platinum (20nm).

Observations. SEI scanning EM demonstrated a complex luminal surface distribution pattern by the membrane-associated differentiation antigen this being

Table 3

A REVIEW OF SEM BIOMEDICAL APPLICATIONS OF THE COLLOIDAL GOLD MARKER SYSTEM

Identifier	Visualizer	Probe (dia. nm)	Cells or tissues	Prep[a]	Coating[b]	References
Marking of surface components						
a) Cell surface antigens						
Yeasts						
-	RA - Mannan	40:50	C.utilis:S.cerevisiae	F	-	Horisberger et al 1976; Horisberger & Rosset 1977a,b
-	RA - C.utilis	40:60	C.utilis:S.cerevisiae	F	-	Horisberger et al 1975, 1976
MCA-acid phosphatase/RAM	SpA-Au	10:15	S. pombe	U	-	Walther et al 1984
Animal cells/tissues						
RA-HT29	RAH-Au	37	Rat erythrocytes	U	+	Goodman et al 1979
RA-HT29	RAH-Au	37	Colon carcinoma cell line HT29	U; F	+	Goodman et al 1979
RA-Line 10	SpA-Au	20:50	Guinea-pig hepato-carcinoma cell lines	U	-	Hoyer et al 1979
-	Bovine Factor VIII-Au	23	Human platelets	U; F	-	Furlan et al 1981
RA-UMA	GAR-Au	45	Rat bladder urothelium	F	+	Hodges et al 1982; Hodges & Kenemans 1982
M-IgM/MCA (D2)	SpA-Au	20:45	Human granulocytes	F	C	de Harven et al 1984
M-IgM/MCA (D2)	SpA-Au	20:40	Human erythrocytes	F	C	Nava et al 1984
M-IgM/MCA (D2)	SpA-Au	15:40	Human leukocytes	F	C	de Harven & Soligo 1986; Soligo et al 1986
-	Fibrinogen-Au	18	Human platelets	U	+:C	Loftus & Albrecht 1983, 1984
-	Fibrinogen-Au	18	Human platelets	U	+:C	Albrecht et al 1986
-	MCA glycoprotein IIb/IIIa fibri-nogen receptor-Au	18	Human platelets	U	+:C	Albrecht et al 1986
Anti-erythrocyte total protein	SpA-Au	5:10:15	Human erythrocytes	F	C	Walther and Müller 1985, 1986
RA-acetyl-cholinesterase	SpA-Au	10:15	Human erythrocytes	F	C	Walther et al 1983, 1984
GA-Fibronectin	RAG-Au	35	Quail neural crest cells	U	+	Sieber-Blum et al 1981
RA-Fibronectin	SAR-Au	45	Human, hamster, mouse fibroblast cell lines	F	C	Trejdosiewicz et al 1981
RA-Fibronectin	SpA-Au	40	Amphibian gastrulae; A.mexicaneum; P.Waltlii	U	+	Darribere et al 1985
RA-Fibronectin	GAR-Au	44	Amphibian gastrulae; X.laevis	F	+	Nakatsuji et al 1985
RA-Fibronectin	SAR-Au	25:40	Human breast epithelial cultures on collagen gel	F	+:C	Peachey & Smolira 1984
RA-Type IV collagen	GAR-Au	25:40	Human breast epithelial cultures on collagen gel	F	+:C	Peachey & Smolira 1984

Identifier	Visualizer	Probe (dia. nm)	Cells or tissues	Prep[a]	Coating[b]	References
b) Cell surface lectins						
Yeasts						
-	BSL-Au	50	S.pombe	U	-	Horisberger & Rosset 1977c; Horisberger et al 1978b
-	Con A-Au	32:50:97	S.cerevisiae C.utilis	U:F	-:+	Horisberger & Rosset 1976, 1977a; Horisberger et al 1976
-	" "	50	Tetrahymena	F	+	Csaba and Madarsz 1979
Con A	Mannan-Au	50	C.utilis	F	-:+	Horisberger & Rosset 1977a,b
-	WGA-BSA-Au	50	S.cerevisiae	F	-	Horisberger et al 1976; Horisberger & Rosset 1976
-	" "	50	S.pombe	F	-	Horisberger et al 1978b
RCA	DC-Au	50	S.pombe	F	-	Horisberger et al 1978b
RCA	guaran-Au	50	S.pombe	F	-	Horisberger et al 1978b
Plants						
-	Con A-Au	n.a.	Tobacco protoplasts	U	+	Burgess & Linstead 1976, 1977
Animal cells/tissues						
-	BSL-Au	50	Human/bovine milk fat globules	U	-	Horisberger et al 1975
-	Con A-Au	26:50	Human/bovine milk fat globules	U	-	Horisberger et al 1977; Horisberger & Rosset 1977b
-	" "	50:75	Human erythrocytes	F	-	Horisberger & Rosset 1977a
-	" "	50:64:75	Rat hepatocytes	F	-	Horisberger et al 1978b; Horisberger & Rosset 1977b
-	Con A-BSA-Au	32:50	Human platelets	F	-	Nurdin et al 1980
Con A	HRP-Au	45	Rat bladder urothelium	F	+	Hodges et al 1982
Con A	"	17	Murine macrophages	U	+	Takata & Hirano 1984
Con A:RA-Con A	GAR-Au	27:36	Mouse bladder urothelium	F	+	Hodges et al 1985
-	PNA-Au	45	Rat bladder urothelium	F	+	Hodges et al 1982
-	" "	50	Rat hepatocytes	U	-	Horisberger et al 1978a
-	" "	25:40	Human breast epithelial cultures on collagen gels	F	+:C	Peachey & Smolira 1984
-	" "	50	Human/bovine milk fat globules	U	-	Horisberger et al 1975
-	RCA-BSA-Au	32:45:50 64	Human erythrocytes	F	-	Horisberger 1979; Horisberger & Tacchini-Vonlanthen 1983
-	" "	32:50	Human platelets	F	-	Nurdin et al 1980
-	" "	32:50	Rat hepatocytes	F	-	Horisberger et al 1978a
-	SBA-Au	26:32:41 50	Human/bovine milk globules	U	-	Horisberger & Rosset 1977b; Horisberger 1979; Horisberger & Tacchini-Vonlanthen 1983
-	"	50:75	Rat hepatocytes	F	-	Horisberger & Rosset 1977b; Horisberger et al 1978a
-	"	35	Human erythrocytes	F	-	Horisberger & Rosset 1977b

(Table 3 continued on next page).

Identifier	Visualizer	Probe (dia. nm)	Cells or tissues	Prep[a]	Coating[b]	References
-	WGA-BSA-Au	26:50	Human/bovine milk fat globules	U	-	Horisberger et al 1977; Horisberger & Rosset 1977b
-	" "	32:50:64 75:97	Human erythrocytes	U:F	-	Horisberger & Rosset 1977a,b; Horisberger 1979; Horisberger & Tacchini-Vonlanthen 1983
-	" "	32:50	Human platelets	F	-	Horisberger et al 1978b; Nurdin et al 1980
-	" "	32:50:64 75	Rat hepatocytes	F	-	Horisberger et al 1978a; Horisberger & Rosset 1977b
-	WGA-Dex-Au	20:30	Chinese hamster ovary cells	U	-	Hicks & Molday 1984

Table abbreviations

a	State of tissue at labelling
F =	Fixed
U =	Unfixed
b + =	Metal coated specimen
c =	Carbon coated specimen
- =	Noncoated specimens
n.a.	Not available
A.Mexicanum	Ambystoma mexicanum
Au	Gold
BSA	Bovine serum albumin
BSL	Bandeiraea simplicifolia lectin
C.utilis	Candida utilis
Con A	Canavalia enisformis lectin
DC	Desialated ceruloplasmin
Dex-Au	Gold-dextran marker
D2	Mouse IgM MCA to normal human ganulocytes
GA-	Goat anti-
GAR	Goat anti-rabbit IgG
HRP	Horseradish peroxidase
MCA	Monoclonal antibody
M-Igm	Mouse IgM antibody
PNA	Arachis hypogea lectin (Peanut agglutinin)
P.Waltlii	Pleurodeles waltilii
RA-	Rabbit anti-
RAG	Rabbit anti-goat IgG
RAH	Rabbit anti-human IgG
RAM	Rabbit anti-mouse IgG
RCA	Ricinus communis lectin
SAR	Sheep anti-rabbit Ig
SBA	Glycine max lectin (Soya bean agglutinin)
S.cerevisiae	Saccharomycecs cerevisiae
SpA	Streptococcus aureus protein A
S.pombe	Schizosaccharamyces pombe
WGA	Triticum vulgaris lectin (Wheatgerm agglutinin)
UMA	Urothelial membrane-associated antigen
X.laevis	Xenopus laevis

a) expressed on the immature (Fig 1) and early differentiating intermediate urothelial cells, b) absent at the mid to late differentiating intermediate stage (Figs 1,3), c) re-expressed as the cells mature (Fig 2) and e) found most abundantly associated with the asymmetric unit membrane of the terminally differentiated superficial cells (Figs 3, 4a,b) thereby demonstrating UMA specificity to be clearly associated with normal urothelial differentiation.

Localization of cell surface antigens (II)

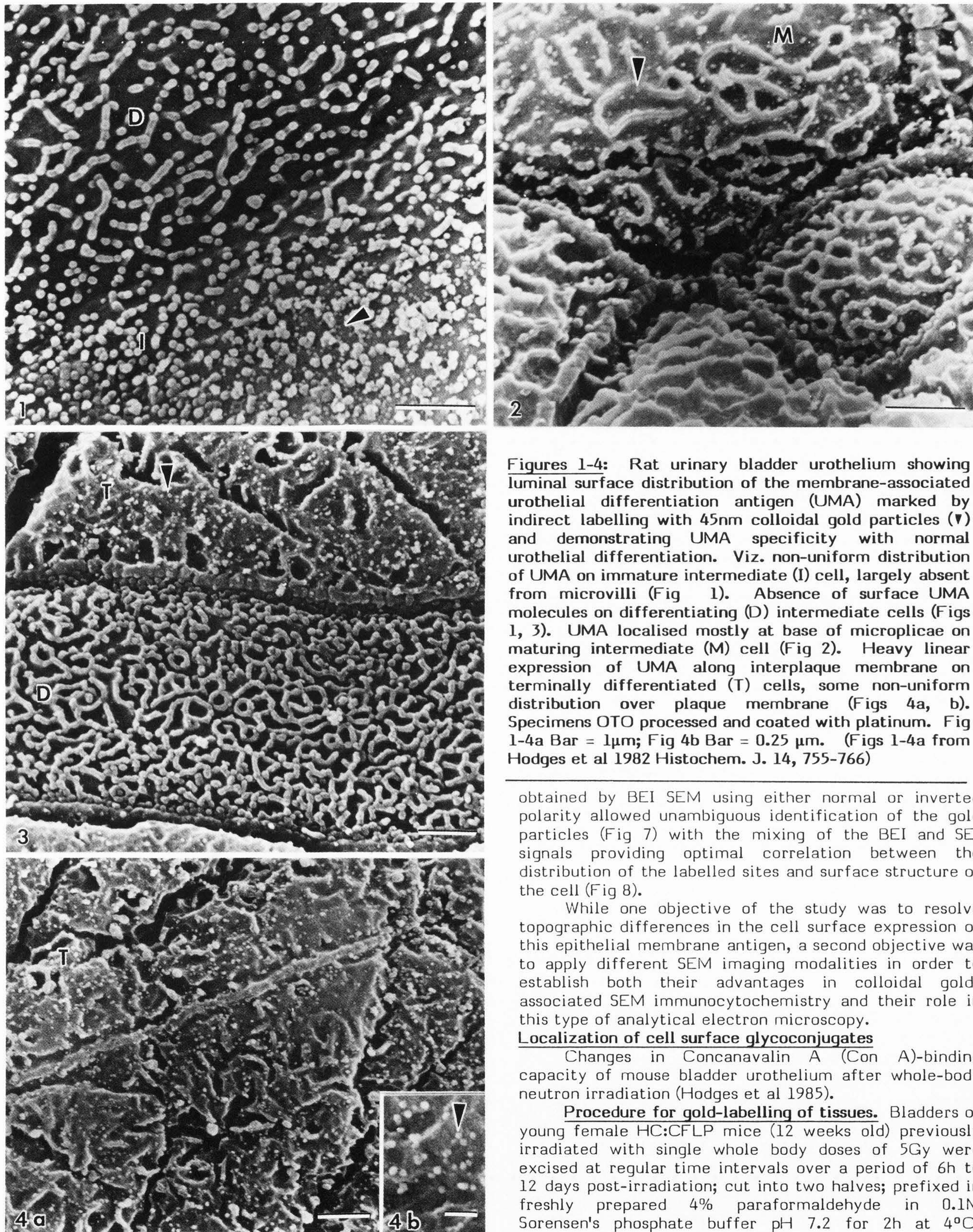
Expression and distribution of a surface epithelial membrane antigen as recognised by an absorbed rabbit antibody (coded L19)(Nicholls et al 1985) on cells of the established and well-differentiated human colon carcinoma-derived HT29 line.

Procedure for gold-labelling of cells. HT29 cells, plated onto teflon-coated glass "Multitest" slides and cultured in 5mM galactose-supplemented E4:RPMI 1640 (1:1) medium (without glucose) containing 5% dialyzed foetal calf serum, were rinsed three times with TBS (see above); prefixed with 4% paraformaldehyde in Sorensen's phosphate buffer pH7.2 for 20 min; washed with 20mM Hepes-buffered medium; incubated with absorbed rabbit antibody L19 (diluted 1:100 in TBS) for 60 min; washed with TBS; reacted with undiluted goat anti-rabbit Ig-gold (30nm dia) probe for 60 min; then washed with TBS; postfixed; and processed for SEM as above with vacuum-evaporated carbon coating replacing the application of a metal coating. All fixation and incubation steps were at ambient temperature and were followed by at least three 5 min washes in buffer or medium as indicated above. Specificity controls included omission of primary antibody.

Procedure for immunofluorescence-labelling of cells. HT29 cells, cultured as above were rinsed with TBS; prefixed with 10% formalin in isotonic PBS (+ Ca^{2+} and Mg^{2+}) for 20 min; washed in TBS (3 x 5 min) then incubated with L19 primary antibody as above; washed in TBS (3 x 5 min); reacted with a fluorescein isothiocyanate-conjugated goat anti-rabbit Ig antiserum (GaR-FITC) (Cappel, Malvern, PA, USA) (diluted 1:20 in TBS) for 30 min; washed in TBS (3 x 5 min), and, mounted on Gelvatol 20/30 (Monsanto, St. Louis, MO, USA)(+ 100mg/ml 1,4 diazabicyclo (2.2.2.) octane -DABCO) (Aldrich Chemical Co., Milwaukee, WI, USA).

Observations. The predominantly speckled pattern of L19 surface labelling observed by immunofluorescence (Fig 5) was clearly detected and amplified by SEM of gold-labelled cells (Figs 6-8).

SEI SEM revealed a non-uniform distribution of gold particles marking the expression of epithelial membrane determinant(s) as recognized by L19 antibody with some evidence of a heavier linear localization on many of the surface microvilli (Fig 6). The results



Figures 1-4: Rat urinary bladder urothelium showing luminal surface distribution of the membrane-associated urothelial differentiation antigen (UMA) marked by indirect labelling with 45nm colloidal gold particles (▼) and demonstrating UMA specificity with normal urothelial differentiation. Viz. non-uniform distribution of UMA on immature intermediate (I) cell, largely absent from microvilli (Fig 1). Absence of surface UMA molecules on differentiating (D) intermediate cells (Figs 1, 3). UMA localised mostly at base of microplicae on maturing intermediate (M) cell (Fig 2). Heavy linear expression of UMA along interplaque membrane on terminally differentiated (T) cells, some non-uniform distribution over plaque membrane (Figs 4a, b). Specimens OTO processed and coated with platinum. Fig 1-4a Bar = 1μm; Fig 4b Bar = 0.25 μm. (Figs 1-4a from Hodges et al 1982 Histochem. J. 14, 755-766)

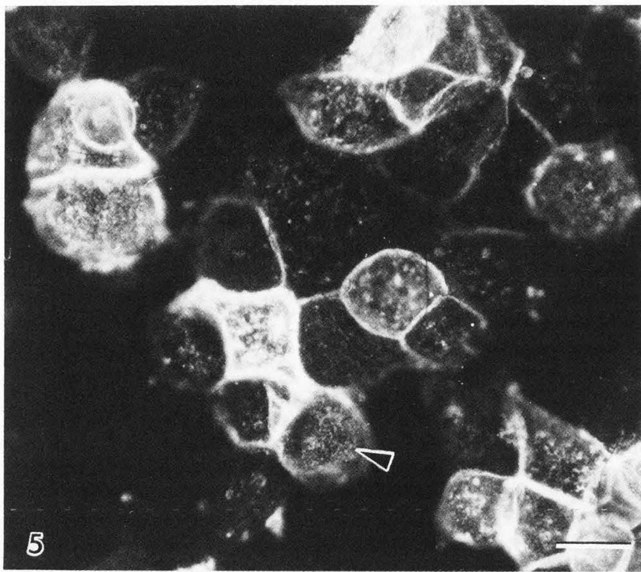
obtained by BEI SEM using either normal or inverted polarity allowed unambiguous identification of the gold particles (Fig 7) with the mixing of the BEI and SEI signals providing optimal correlation between the distribution of the labelled sites and surface structure of the cell (Fig 8).

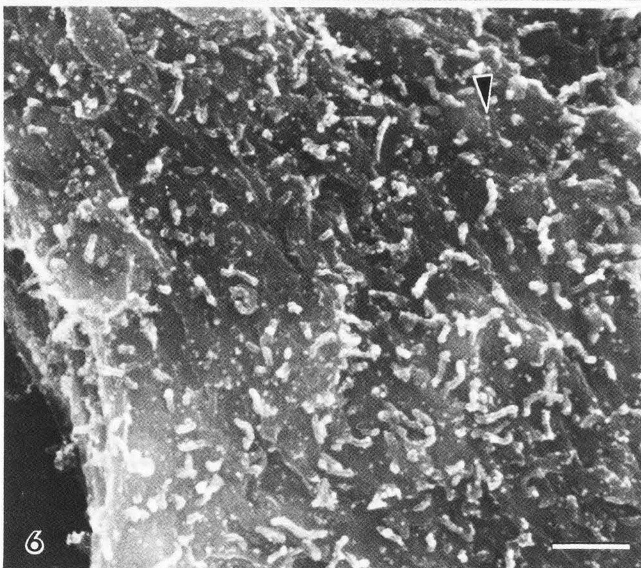
While one objective of the study was to resolve topographic differences in the cell surface expression of this epithelial membrane antigen, a second objective was to apply different SEM imaging modalities in order to establish both their advantages in colloidal gold-associated SEM immunocytochemistry and their role in this type of analytical electron microscopy.

Localization of cell surface glycoconjugates

Changes in Concanavalin A (Con A)-binding capacity of mouse bladder urothelium after whole-body neutron irradiation (Hodges et al 1985).

Procedure for gold-labelling of tissues. Bladders of young female HC:CFLP mice (12 weeks old) previously irradiated with single whole body doses of 5Gy were excised at regular time intervals over a period of 6h to 12 days post-irradiation; cut into two halves; prefixed in freshly prepared 4% paraformaldehyde in 0.1M Sorensen's phosphate buffer pH 7.2 for 2h at 4°C; reactive aldehyde groups blocked by incubation with 0.2M glycine in PBS for 30 min; the bladder halves further dissected into $3mm^2$ pieces; the tissue pieces

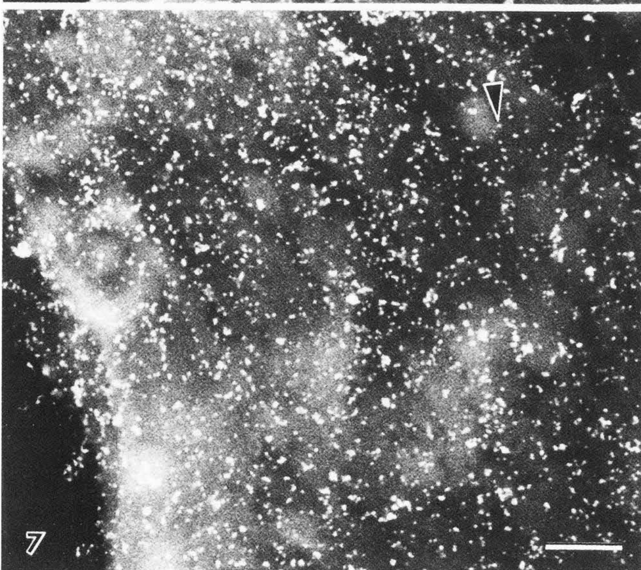


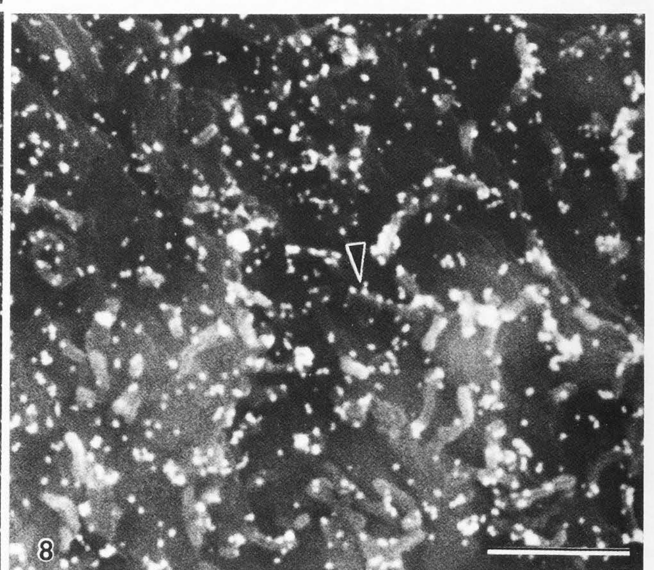


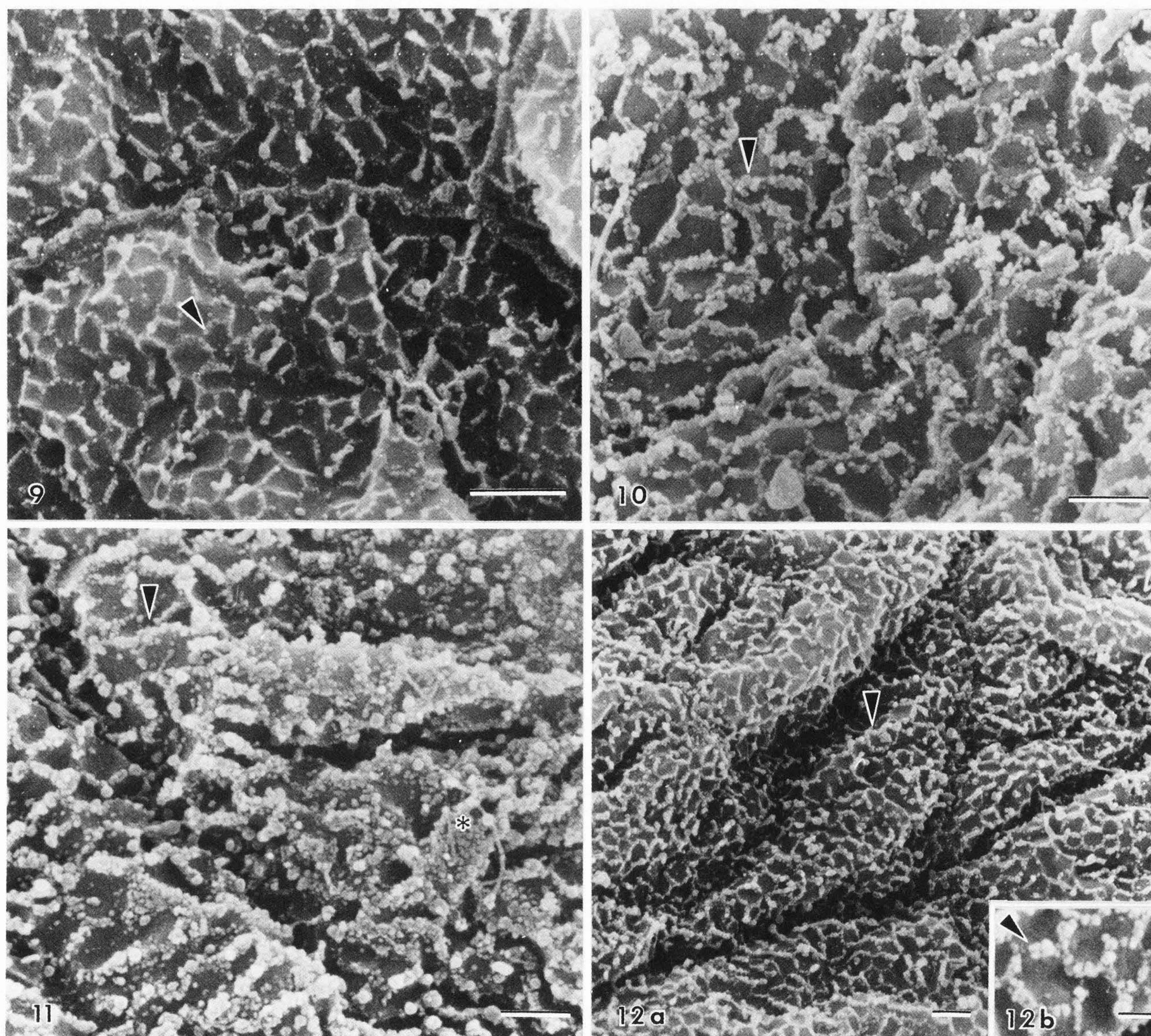
distributed into each of a 6-well (3cm dia) Limbro dish containing TBS; and then exposed to a three-layer labelling procedure. All reactions were performed at ambient temperature with each step followed by at least three washes in TBS (see above) or in TBS supplemented with 1mM $MnCl_2$ and 1mM $CaCl_2$ (TBS-S). The tissues were incubated with Con A (100ug/ml in TBS-S) for 60 min at ambient temperature; washed in TBS; covered with affinity-purified rabbit anti-Con A (100ug/ml in TBS) for 60 min; washed in TBS; and reacted with undiluted affinity purified goat anti-rabbit Ig-gold (27 or 36nm dia) probe for a further 60 min; then washed in TBS; post fixed and processed for SEM as described above. Specificity controls for Con A labelling included the omission of Con A or the 1:1 addition of 0.25M specific inhibitor (d-methyl-D-mannoside) to the Con A solution.

Observations. Con A receptor sites were clearly identifed on the luminal surface of the superficial cells of bladder urothelium by SEI scanning EM of gold-labelled tissues (Figs 9-12b). The urothelial luminal membrane labelling pattern in bladders of non-irradiated animals showed the expression of membrane mannose- and glucose-rich carbohydrate moieties to be essentially restricted to the regions of the interplaque membranes (Fig 9). By contrast, progressively prominent lectin-binding to the luminal asymmetric unit membrane (AUM) occurred over the 6h to 5 day post-irradiation time period

Figures 5-8: Cell-surface localization of L19 antibody on cultures of human colon-derived HT29 cells. Immunofluorescence establishes cell-surface reaction as a punctate pattern of variable intensity with often a stronger reaction at the areas of cell-cell contact (Fig 5). SEI SEM reveals a non-uniform distribution of the L19 reactive epithelial membrane antigen marked by indirect labelling with 30nm gold particles (▼) (Fig 6) unambiguously identified by BEI SEM (used in normal polarity mode) (Fig 7) and presenting evidence of a heavier linear concentration along many of the surface microvilli by mixed SEI-BEI (normal polarity) imaging (Fig 8). Specimens secondarily fixed with osmium tetroxide (1%) and coated with carbon. Fig 5 Bar = 10 µm; Figs 6-8 Bar = 1µm.







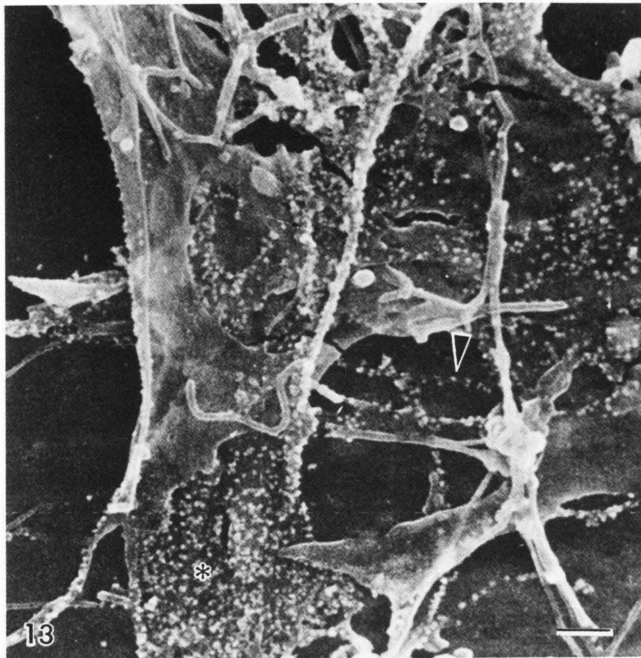
Figures 9-12 illustrate representative luminal surface distributions of Con A receptor sites marked by indirect labelling with 30nm gold particles on the superficial terminally-differentiated urothelial cells of urinary bladders from sham-irradiated control mice (Fig 9) and whole-body 5Gy neutron-irradiated mice (Figs 10-12b). SEI SEM demonstrates discrete localization of Con A receptor sites to the interplaque region of the AUM of non-irradiated bladder urothelium (Fig 9); prominent Con A-binding to the interplaque regions of bladder urothelium 1 day post-irradiation (Fig 10); expression of Con A receptor sites over both plaque (*) and interplaque (▼) areas of the luminal plasma membrane of bladder urothelium 5 days post-irradiation (Fig 11); and reversion of the Con A binding pattern toward that of the control group by 7 days post-irradiation (Fig 12a, b). Specimens OTO processed and coated with platinum. Figs 9-12b Bar = 1μm. (Figs 9-12b from Hodges et al 1985 Scanning Electron Microsc. 1985; IV: 1603-1614).

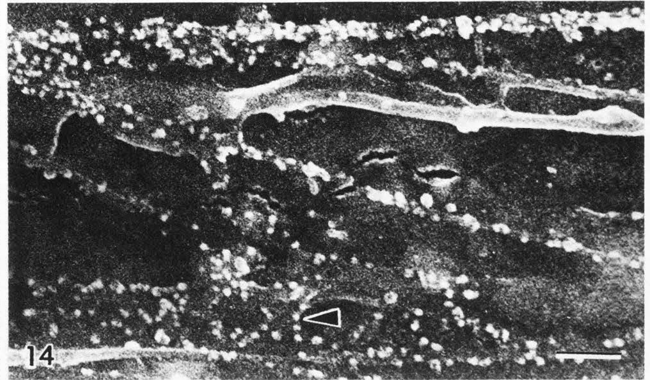
with expression of Con A receptor sites over both plaque (AUM) and interplaque areas (Figs 10,11): this atypia in density and distribution pattern of mannose- and glucose-rich surface glycoconjugates showed partial regression over the following 5 to 12 day post-irradiation time period (Figs 12a,b).

Localization of extracellular components

Expression and distribution of cell surface fibronectin in single and mixed epithelial-fibroblast cell cultures (Trejdosiewicz et al 1981).

Procedure for gold labelling of cells. Monolayer cell cultures grown on Melanex coverslips were washed; prefixed with 1% paraformaldehyde in Sorensen's phosphate buffer pH7.2 for 15 min; incubated with affinity purified rabbit anti-fibronectin (diluted 1/100 in PBS-BSA) for 45-60 min; washed; reacted with undiluted sheep anti-rabbit Ig-gold (45nm dia) probe; then washed; post fixed; and processed for SEM as described above, but with a coating of vacuum-evaporated carbon being used instead of platinum. All fixation and labelling steps





Figures 13-14: Cell-surface distribution by fibronectin in a culture of the Hel17 fibroblasts marked by indirect labelling with 45nm gold particles (▼). SEI (Fig 13) and BEI (normal polarity) (Fig 14) SEM shows the gold particles located and aligned along fibronectin strands (▼) and over occasional dense mats of fibronectin (*). Specimens OTO processed and coated with carbon. Fig 13 Bar = 1µm; Fig 14 Bar = 0.5 µm. (Figs 13, 14 from Trejdosiewicz et al 1981 J. Microscopy 123, 227-236)

were performed at ambient temperature; each step was followed by at least three washes of 5-10 min in phosphate-buffered saline containing 1mg/ml bovine serum albumin (PBS-BSA). Specificity controls consisted of omission of first antibody and the use of irrelevant first antibody (keratin).

Observations. SEI SEM clearly detected the gold particles located and aligned on the extracellular network of fibronectin (Fig 13), and conformed with fibronectin labelling patterns established by immunofluorescence. The amplification of such labelling patterns provided by SEM of gold-labelled cells allowed the cellular origin of the fibronectin strands to be more clearly identified and showed fibronectin expression in the mixed epithelial-fibroblast cultures to be restricted to cells of mesenchymal origin. The enhanced contrast provided by BEI scanning EM was clearly established (Fig 14) and emphasized the point that BEI SEM could facilitate discrimination between true immuno-negative and very weak immuno-positive specimens.

Localization of intracellular antigens

Cytokeratin expression and organization in cells of the well-differentiated human urinary bladder carcinoma cell lines RT112 and RT4 (see Masters et al 1986 for description and references to cell lines).

Procedure for gold-labelling of cells

Group A: RT112 cells, plated onto teflon-coated glass "Multitest" slides and cultured in E4:RPMI 1640 (1:1) medium containing 5% foetal calf serum, were washed with 20mM Hepes-buffered medium; fixed in a freshly prepared 1:1 mixture of methanol and acetone for 1 min at ambient temperature with gentle agitation; followed by gradual replacement of the methanol:acetone mixture with 20mM Hepes-buffered medium; then washed (3-5 times) with 20mM Hepes-buffered medium prior to labelling. The cells were incubated with an anti-keratin monoclonal antibody LdS 103 (undiluted hybridoma culture supernatant) obtained after PEG-induced fusion of splenocytes of mice immunized with cytoskeletons of the human urothelial RT4 cell line (Southgate and Trejdosiewicz, in preparation) for 60 min at ambient temperature; washed (3 x 5 min) in TBS; reacted with undiluted affinity-purified goat anti-mouse Ig-gold (30 nm dia) probe for a further 60 min; then washed in TBS (3 x 5 min); and processed for SEM as above, followed by application of a carbon-coating.

Group B: RT112 cells cultured as above (see Group A) were rinsed in PHEM buffer (60mM PIPES buffer in distilled water containing 25mM Hepes, 10mM EGTA, 1mM $MgCl_2$ and buffered to pH 6.9 with N/1 NaOH); incubated with PHEM buffer containing 0.15% Triton X-100 and 0.6M potassium iodide for 5 sec; washed with 20mM Hepes-buffered medium; then methanol: acetone fixed, diluted into aqueous phase, labelled, and processed for SEM as above.

Group C: RT4 cells cultured as above (see Group A) were rinsed in PHEM buffer (see above Group B); incubated with PHEM-4M glycerol buffer containing 0.15% Triton X-100 for 5min; washed with PHEM; then paraformaldehyde-fixed; incubated with an anti-keratin monoclonal antibody LAS86 (undiluted hybridoma culture supernatant) containing 0.1% sodium azide and gold-labelled as above (see Group A); then processed for SEM followed by application of a carbon-coating.

Procedure for immunofluorescence-labelling of cells

Group A: RT112 cells were cultured, methanol:acetone-fixed, diluted into aqueous phase, and labelled with primary antibody as above; washed with TBS; then reacted with tetraethyl rhodamine isothiocyanate (TRITC) (Cappell, Malvern, PA, USA) conjugate of goat anti-mouse (GaM) Ig (diluted 1:20 in TBS) for 30 min; washed with TBS (3 x 5 min), and mounted on Gelvatol 20/30 (+ 100 mg/ml DABCO).

Group B: RT112 cells were cultured; rinsed with PHEM buffer; detergent-extracted; washed with 20mM Hepes-buffered medium; then methanol:acetone fixed and labelled with primary antibody as above; then reacted with TRITC-GaM Ig; washed with TBS; and mounted on Gelvatol 20/30 (+ 100mg/ml DABCO).

Observations. Immunofluorescence imaging of cytokeratin localization patterns showed these to differ with chemical permeabilization procedure. Fine characteristic tonofilament arrays extending throughout the cytoplasm were displayed by RT112 cells exposed to protein precipitation and lipid extraction consequent to methanol-acetone treatment (Fig 15). By contrast, cytokeratin labelling of RT112 cells detergent-extracted with Triton X-100 revealed an apparent loss of fine filaments visualizing instead a loose network of relatively thicker filaments typically displaying a "beaded" aspect (Fig 16).

Comparative SEI and BEI SEM (Figs 17-21) established that cytokeratin tonofilament arrays may be readily visualized at low-power magnifications by BEI imaging (cf Figs 18;20) while, following immuno-gold labelling, arrays of cytokeratin filaments could be identified by SEI SEM in areas of the cell devoid of plasma membrane (Fig 21). These observations emphasized, however, the constraint imposed by intra-cellular SEI visualization of gold markers in that intra-cellular entities of similar shape and size could prove difficult to distinguish from the gold particles (Fig 21). By contrast, localization of cytokeratin proteins were clearly detected and amplified by BEI of gold-labelled cells. BEI SEM, used either in normal or inverted polarity mode, allowed the unambiguous identification of gold particles aligned along keratin filaments either exposed in cells devoid of plasma membrane, or present as subsurface structures in cells retaining their plasma membrane (Figs 22,23).

The primary objective of this work is aimed at defining changes in intermediate filament localization patterns and keratin epitope expression associated with different chemical permeabilization procedures. But a secondary objective reported in this study was to establish that the enhanced contrast provided by BEI SEM could clearly identify sub-surface intracellular gold-labelled target sites thereby further enhancing the analytical capabilities of the SEM in the area of immunocytochemistry, notably in making possible a precise correlation between cell surface architecture and presence and distribution of specific intracellular antigens.

Conclusions

Colloidal gold, since its introduction as a cyto-chemical marker in the early 1970s, has proven to be a versatile and efficient immunocytochemical labelling probe both for light microscopy and for transmission and scanning electron microscopy. From the examples of applications cited in this review three principal conclusions can be drawn. First, that colloidal gold represents a unique, widely applicable marker system for the correlative microscopic visualization of cell and tissue constituents. Second, that gold-labelled reagents are effective probes which can be readily exploited for SEM immunocytochemistry. Third, that as a consequence, SEM can both offer a unique system for positive identification, localization and quantification of cell surface and intracellular target molecules, and a promising approach to the study of specific transmembrane molecular interrelationships. A further extension in such applications can be expected from the use of backscattered electron-imaging, which, combined with secondary electron-imaging provides for an optimal correlation between labelled sites and ultrastructure.

With the recent developments discussed here, notably use of the colloidal gold marker system, of BEI SEM, and of diverse biological specimen preparation options, molecular information at higher orders of cell surface and intracellular structure can now be readily obtained by the SEM thereby establishing the framework for further expansion of SEM imunocytochemistry as a routine analytical procedure.

References

Albrecht RM, Oliver JA, Loftus JC. (1986). Observation of colloidal gold labelled platelet surface receptors and the underlying cytoskeleton using high voltage electron microscopy and scanning electron microscopy. in: The Science of Biological Specimen Preparation". M Mueller, R P Becker, A Boyde, J J Wolosewick (eds), SEM Inc, AMF O'Hare (Chicago) IL 60666. pp. 185-193.

Autrata R, Walther P, Kriz S, Müller M. (1986). A BSE scintillation detector in the (S)TEM. Scanning **8**, 3-8.

Becker RP, Geoffroy JS. (1981). Backscattered electron imaging for the life sciences introduction and index to applications - 1961 to 1980. Scanning Electron Microsc. 1981; IV:195-206.

Becker RP, & Sogard M. (1979). Visualization of subsurface structures in cells and tissues by back-scattered electron imaging. Scanning Electron Microsc. 1979; II:835-870.

Beesley JE. (1985). Colloidal gold: a new revolution in marking cytochemistry. Proc. Royal Microscopical Society **20/4**, 187-196.

Bell Jr PB. (1981). The application of scanning electron microscopy to the study of the cytoskeleton. Scanning Electron Microsc. 1981; II:139-157.

Bell PB, Stark-Vancs VI. (1983). Some factors affecting the preservation of the cytoskeleton of cells in culture for scanning electron microscopy. Scanning Electron Microsc. 1983; I:351-359.

Borwein B. (1985). Scanning electron microscopy in retinal research. Scanning Electron Microsc. 1985; I:279-301.

Boyde A. (1975). A method for the preparation of cell surfaces hidden within bulk tissue for examination in the SEM. Scanning Electron Microsc. 1975:295-301.

Boyde A, Wood C. (1969). Preparation of animal tissue for surface scanning electron microscopy. J.Microscopy, **90**, 221-249.

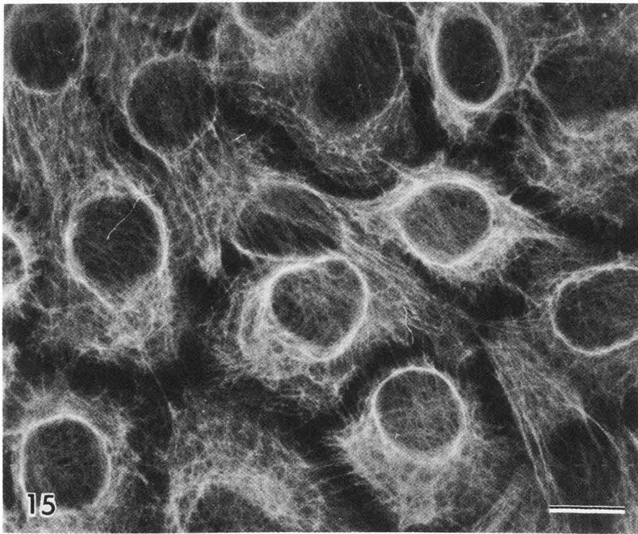
Brown SS, Revel JP. (1978). Cell surface labelling for the scanning electron microscope. in: Advanced Techniques in Biological Electron Microscopy, Vol 2. J H Koehler (ed.) Springer Verlag, Berlin-Heidelberg-New York, pp. 65-88.

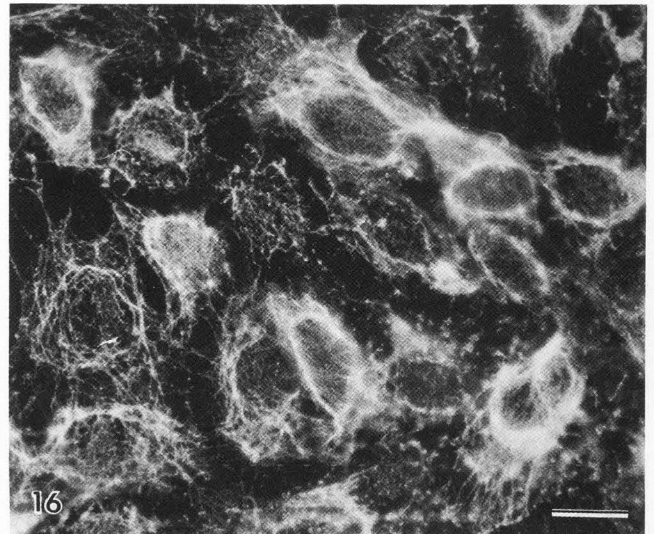
Bullock GR, Petrusz P (eds). (1982). Techniques in Immunocytochemistry, Vol I, Academic Press, London and New York.

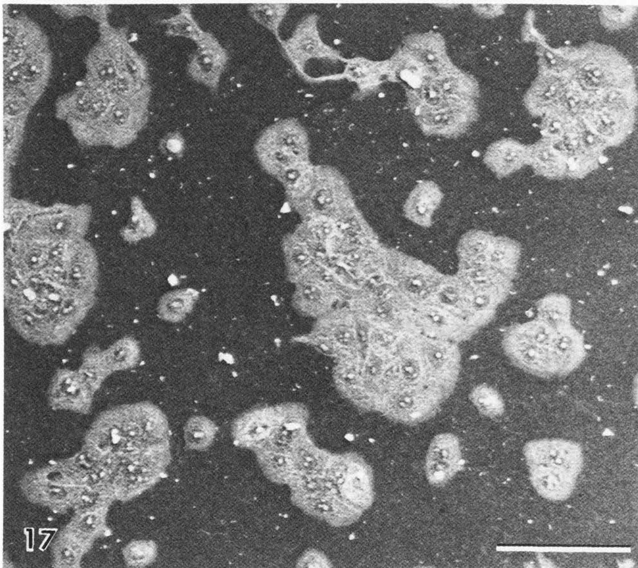
Bullock GR, Petrusz P (eds). (1983). Techniques in Immunocytochemistry, Vol II, Academic Press, London and New York.

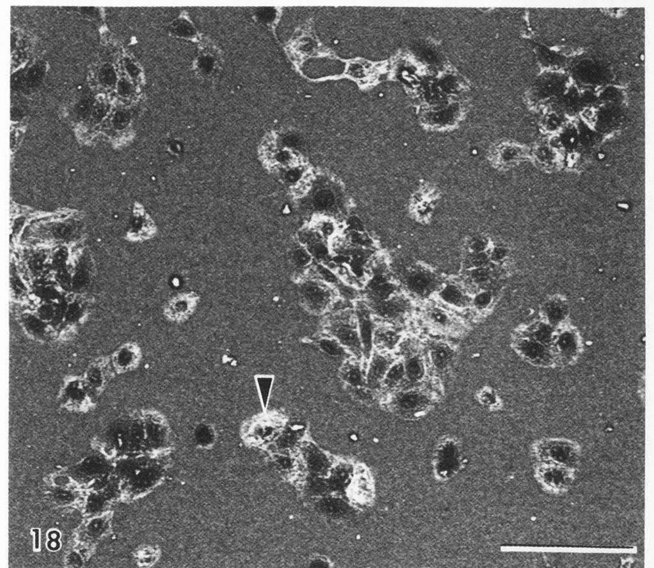
Bullock GR, Petrusz P (eds). (1985). Techniques in Immunocytochemistry, Vol III, Academic Press, London and New York.

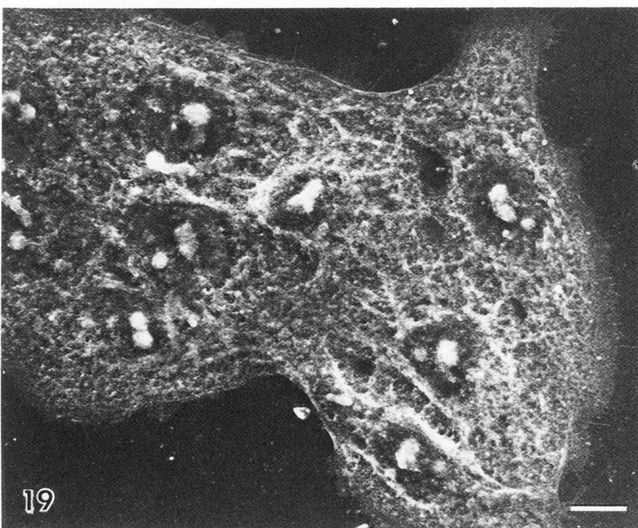
Burgess J, Linstead PJ. (1976). Ultrastructural studies of the binding of Concanavalin A to the plasmalemma of higher plant protoplasts. Planta, **130**, 79-83.

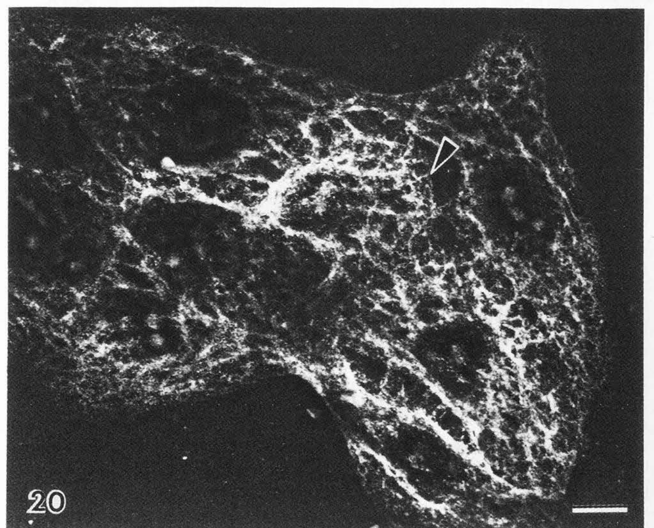

15

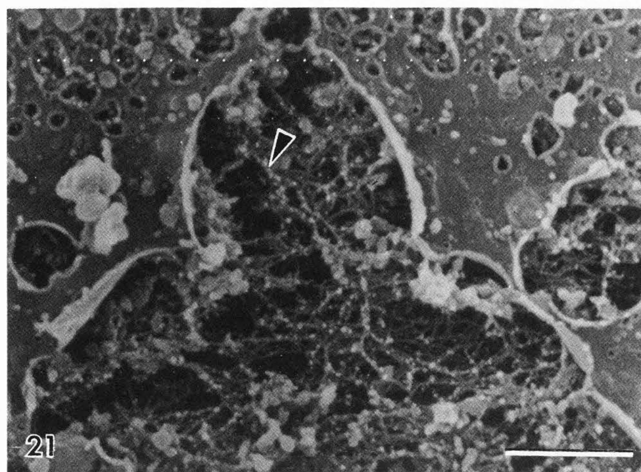

16

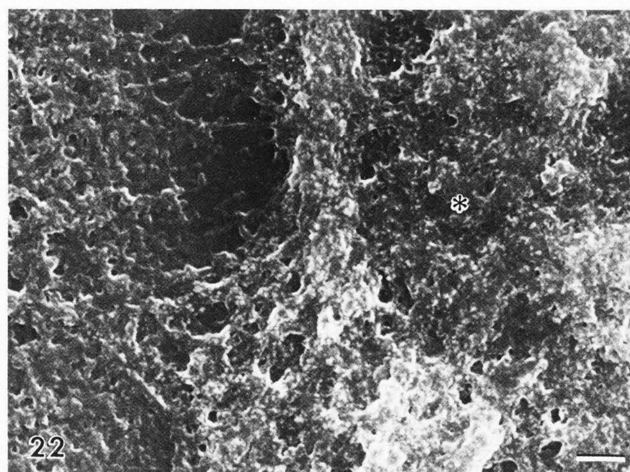

17

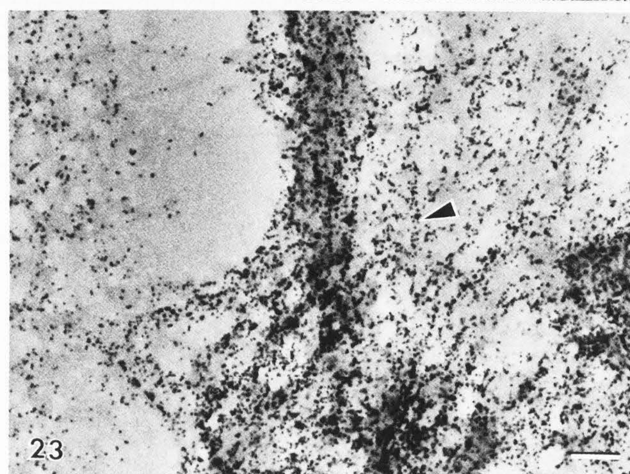

18


19


20







Figures 15-23 illustrate characteristic intracellular immuno-localization patterns seen with anti-keratin LdS 103 monoclonal antibody on methanol-acetone treated (Figs 15, 17-20, 22-23) or detergent-extracted (Figs 16, 21) RT112 (Figs 15-20, 22-23) or RT4 (Fig 21) cells. Immunofluorescence establishes dense fibrillar intermediate filament arrays in RT112 cells permeabilized with a 1:1 methanol-acetone mixture (Fig 15) and a looser "speckly" filamentous network in RT112 cells extracted with Triton X-100 (Fig 16). Comparative SEI (Figs 17, 19) and BEI (normal polarity) (Figs 18, 20) SEM provides low-power confirmation of the unambiguous identification of the "cytoskeletal" localization (▾) that may be established in individual cells or cell clumps by BEI imaging. Fig 21 illustrates arrays of cytokeratin filaments seen with LAS86 monoclonal antibody and marked by indirect labelling with 30nm gold particles (▾) as revealed by SEI SEM in plasma membrane-free areas of detergent-extracted cells. Comparative SEI (Fig 22) and BEI (reverse polarity) (Fig 23) SEM establishes the potential of sub-surface visualization of cytoskeletal arrays by BEI imaging: Fig 22-SEI SEM of cell surface (*); Fig 23 - sub-surface localization of cytokeratin filaments marked by linear arrays of gold particles (▾). Specimens secondarily fixed in osmium tetroxide (1%) and coated with carbon. Figs 15, 16, 19, 20 Bar = 10 µm; Figs 17, 18 Bar = 100 µm; Figs 21-23 Bar = 1 µm.

Burgess J, Linstead PJ. (1977). Membrane mobility and the Concanavalin A binding system of the plasmalemma of higher plant protoplasts. Planta, **136**, 253-259.

Carr KE, Hodges GM, Maxwell W. (1987). Etching techniques for electron microscopy. J.Elect.Micr.Tech. (in press).

Csaba G, Madarsz B. (1979). Localization of Concanavalin A binding sites in Tetrahymena by scanning electron microscopy. Experientia, **35**, 1181-1182.

Darribere T, Boulekbache H, Shi DL, Boucaut JC. (1985). Immuno-electron-microscopic study of fibronectin in gastrulating amphibian embryos. Cell Tissue Res., **239**, 75-80.

De Harven E, Soligo D. (1986). Scanning electron microscopy of cell surface antigens labelled with colloidal gold. Am.J.Anat., **17**, 277-287.

De Harven E, Leung R, Christensen H. (1984). A novel approach for scanning electron microscopy of colloidal gold-labelled cell surfaces. J.Cell.Biol., **99**, 53-57.

De Mey J. (1983). Colloidal gold probes in immunocytochemistry. in: Immunocytochemistry. Practical applications in pathology and biology. J M Polak, S van Norden (Eds), Wright-PSG, Bristol, pp. 82-112.

De Petris S. (1978). Immunoelectron microscopy and immunofluorescence in membrane biology. in: Methods in Membrane Biology, Vol 9, E D Korn (ed)., Plenum Press, New York and London, pp 1-201.

Echlin P. (1981). Recent advances in specimen coating techniques. Scanning Electron Microsc. 1981; I: 79-80.

Furlan M, Horisberger M, Perret BA, Beck EA. (1981). Binding of colloidal gold granules, coated with bovine factor VIII, to human platelet membranes. Br.J.Haematology, **48**, 319-324.

Goodman SL, Hodges GM, Trejdosiewicz LK, Livingston DC. (1979). Colloidal gold probes. A further evaluation. Scanning Electron Microsc. 1979; III:619-628.

Goodman SL, Hodges GM, Livingston DC. (1980). A review of the colloidal gold marker system. Scanning Electron Microsc. 1980; II:133-146.

Goodman SL, Hodges GM, Trejdosiewicz LK, Livingston DC. (1981). Colloidal gold markers and probes for routine application in microscopy. Microscopy **123**, 201-213.

Handley DA, Chien S. (1983). Colloidal gold: a pluripotent receptor probe. Proc.Soc.Exp.Biol.Med. **174**, 1-11.

Hicks D, Molday RS. (1984). Analysis of cell labelling for scanning and transmission electron microscopy, in: The Science of Biological Specimen Preparation, J-P Revel, T Barnard, G H Haggis (eds)., SEM Inc., AMF O'Hare (Chicago) IL 60666, pp203-219.

Hodges GM, Kenemans P. (1982). Diagnostic applications to oncology. in: Atlas of Human Reproduction by Scanning Electron Microscopy, E.S.A. Hafez, P Kenemans (eds), MTP Press Ltd, Lancaster UK, pp 325-327.

Hodges GM, Smolira MA, Trejdosiewicz LK. (1982). Urothelium-specific antibody and lectin surface mapping of bladder urothelium. Histochem.J., **14**, 755-766.

Hodges GM, Smolira MA, Livingston DC. (1984). Scanning electron microscope immunocytochemistry in practice. in: Immunolabelling for Electron Microscopy, J M Polak, I M Varndell (eds), Elsevier, Amsterdam, pp189-234.

Hodges GM, Carr KE, Hume SP, Marigold JCL, Southgate J, Marshall JF. (1985). Changes in surface structure and Concanavalin A binding capacity of urothelium in the mouse bladder after whole-body neutron irradiation. Scanning Electron Microsc. 1985; IV:1603-1614.

Hollenberg MJ, Erickson AM. (1973). The scanning electron microscope - potential usefulness to biologists. J.Histochem Cytochem, **21**, 109-130.

Horisberger M. (1979). Evaluation of colloidal gold as a cytochemical marker for transmission and scanning electron microscopy. Biol. Cellul. **36**, 253-258.

Horisberger M. (1981). Colloidal gold: a cytochemical marker for light and fluorescent microscopy and for transmission and scanning electron microscopy. Scanning Electron Microsc. 1981; II: 9-31.

Horisberger M. (1985). The gold method as applied to lectin cytochemistry in transmission and scanning electron microscopy. in: Techniques in Immunocytochemistry, Vol 3, G Bullock, P Petrusz (eds). Academic Press, London and New York, pp 155-178.

Horisberger M, Rosset J. (1976). Localization of wheat germ agglutinin receptor sites on yeast cells by scanning electron microscopy. Experientia **32**, 998-1000.

Horisberger M, Rosset J. (1977a) Colloidal gold, a useful marker for transmission and scanning electron microscopy. J. Histochem. Cytochem., **25**, 295-305.

Horisberger M, Rosset J. (1977b). Gold granules a useful marker for SEM. Scanning Electron Microsc. 1977; II: 75-82.

Horisberger M, Rosset J. (1977c). Localization of -galactomannan on the surface of Schizosaccharomyces pombe cells by scanning electron microscopy. Arch.Microbiol., **112**, 123-126.

Horisberger M, Tacchnini-Vonlanthen M. (1983). Stability and steric hindrance of lectin-labelled gold markers in transmission and scanning electron microscopy. in: Lectins, Vol. 3, T.C. Bog-Hansen, G.A. Spengler (eds)., De Gruyter, Berlin, pp. 189-197.

Horisberger M, Rosset J, Bauer H. (1975). Colloidal gold granules as markers for cell surface receptors in scanning electron microscopy. Experientia **31**, 1147-1149.

Horisberger M, Rosset J, Bauer H. (1976). Localization of mannan at the surface of yeast protoplasts by scanning electron microscopy. Arch. Microbiol. **109**, 9-14.

Horisberger M, Rosset J, Vonlathen M. (1977). Location of glycoproteins on milk fat globule membranes by scanning and transmission electron microscopy using lectin-labelled gold granules. Exp.Cell.Res., **109**, 361-369.

Horisberger M, Vonlanthen M, Rosset J. (1978a). Localization of -galactomannan and wheat germ agglutinin receptors in Schizosaccharomyces pombe. Arch. Microbiol., **119**, 107-111.

Horisberger M, Rosset J, Vonlanthen M. (1978b). Location of lectin receptors on rat hepatocytes by transmission and scanning electron microscopy. Experientia **34**, 274-276.

Hoyer LC, Bucana C. (1982). Principles of Immunoelectron Microscopy. in: Antibody as a Tool, J J Marchalonis, G W Warr (eds), J Wiley and Sons Ltd, Chichester, New York, pp 233-271.

Hoyer LC, Lee JC, Bucana C. (1979). Scanning immunoelectron microscopy for identification and mapping of two or more antigens on cell surfaces. Scanning Electron Microsc. 1979; III: 629-636.

Kraehenbuhl JP, Racine L, Griffiths GW. (1980). Attempts to quantitate immunocytochemistry at the electron microscope level. Histochem.J., **12**, 317-332.

LoBuglio AF, Rinehart JJ, Balcerzak SP. (1972). A new immunologic marker for scanning electron microscopy. Scanning Electron Microsc. 1972; 313-320.

Loftus JC, Albrecht RM. (1984). Redistribution of the fibrinogen receptor of human platelets after surface activation. J.Cell Biol. **99**, 822-829.

Loftus JC, Albrecht RM. (1983). Use of colloidal gold to examine fibrinogen binding to human platelets. Scanning Electron Microsc. 1983; IV: 1995-1999.

Lucocq JM, Roth J. (1985). Colloidal gold and colloidal silver-metallic markers for light microscopic histochemistry. in: Techniques in Immunocytochemistry, Vol 3, G R Bullock, P Petrusz (eds), Academic Press, London, pp 204-236.

Masters JRW, Hepburn PJ, Walker L, Highman WJ, Trejdosiewicz KK, Povey S, Parker M, Hill BT, Riddle PR, Franks LM. (1986). Tissue culture model of transitional cell carcinoma: characterization of twenty-two human urothelial cell lines. Cancer Res. **46**, 3630-3636.

Molday RS. (1983). Labelling of cell surface antigens for SEM. in: Techniques in Immunocytochemistry, Vol 2, G R Bullock, P Petrusz (eds). Academic Press, London, p 117-154.

Molday RS. (1977). Cell surface labelling techniques for SEM. Scanning Electron Microsc. 1977; II: 59-74.

Molday RS, Maher P. (1980). A review of cell surface markers and labelling techniques for scanning electron microscopy. Histochem.J. **12**, 273-315.

Murphy JA. (1978). Non-coating techniques to render biological specimens conductive. Scanning Electron Microsc. 1978; II:175-194.

Murphy JA. (1980). Non-coating techniques to render biological specimens conductive/1980 update. Scanning Electron Microsc. 1980; I:209-220.

Nakatsuji N, Smolira MA, Wylie CC. (1985). Fibronectin visualised by scanning electron microscopy immunocytochemistry on the substratum for cell migration in Xenopus laevis gastrulae. Devel. Biology. **107**, 264-268.

Nava MT, Soligo D, Pozzoli E, Lambertenghi-Deliliers G. (1984). Back-scattered electron imaging for immunogold viewing by scanning electron microscopy. J.Immunological Methods, **70**, 269-271.

Nemanic M. (1975). On cell surface labelling for the SEM. Scanning Electron Microsc. 1975: 342-349.

Nicholls RJ, Southgate J, Trejdosiewicz LK. (1985). Antibodies to normal human colon membranes: preparation, characterization and tissue distribution. Histochem. J. **17,** 717-729.

Nurdin AT, Horisberger M, Savariau E, Caen JP. (1980). Visualization of lectin binding sites on the surface of human platelets using lectins absorbed to gold granules. Experientia, **36,** 1215-1217.

Oatley C W. (1981). Detectors for the Scanning Electron Microscope. J.Phys.E. Sci. Instr. **14,** 971-976.

Osborn M, Weber K. (1982). Immunofluorescence and immunocytechemical procedures with affinity purified antibodies: tubulin-coating structures. Methods Cell Biol., **24,** 98-132.

Peachey LA, Smolira MA. (1984). The use of colloidal gold complexes in an ultrastructural study of lectin-binding sites and matrix deposition of normal human primary breast epithelium on collagen gels. Histochem. J, **16,** 819-834.

Peters K-R. (1984). Continuous ultrathin metal films. In: Science of Biological Specimen Preparation, J-P Revel, T Barnard, G H Haggis (eds). SEM Inc, AMF O'Hare (Chicago), IL 60666, pp221-231.

Peters K-R. (1985). Working at higher magnifications in scanning electron microscopy with secondary and backscattered electrons on metal coated biological specimens and imaging macromolecular cell membrane structures. Scanning Electron Microsc. 1985; IV: 1519-1544.

Polak JM, Varndell IM, (eds). (1984). Immunolabelling for Electron Microscopy, Elsevier, Amsterdam.

Polliack A, Gamliel H. (1983). Surface immunocytochemistry of lymphocytes using scanning electron microscopy. in: Biomedical Research Applications of Scanning Electron Microscopy. Vol 3, G M Hodges, K E Carr (eds), Academic Press, London and New York, pp 31-77.

Reimer L. (1984). Electron signal and detector strategy. in: "Electron beam interactions with solids", (eds) Kyser DF, Newbury DE, Niedrig H, Shimizu R. SEM Inc, AMF O'Hare (Chicago) IL 60666, pp 299-310.

Reimer L, Volbert B, Bracker P. (1979). STEM semiconductor detector for testing SEM quality parameters. Scanning **2,** 96-103.

Roth J. (1982). The protein-A gold (pAg) technique-a qualitative and quantitative approach for antigen localization on thin sections. in: Techniques in Immunocytochemistry, Vol 1, G R Bullock and P Petrusz (eds). Academic Press, London and New York, pp 108-133.

Roth J. (1983). The colloidal gold marker system for light and electron microscopic cytochemistry. in: Techniques in Immunocytochemistry, Vol 2, G R Bullock, P Petrusz (eds), Academic Press, London and New York, pp 217-284.

Sieber-Blum M, Sieber F, Yamada KM. (1981). Cellular fibronectin promotes adrenergic differentiation of quail neural crest cells in vitro. Expl. Cell. Res. **133,** 285-295.

Soligo D, Lampen B, de Harven E. (1981). Back-scattered electron imaging of human leukocytes. Scanning Electron Microsc. 1981; II: 95-103.

Soligo D, de Harven E, Nava MT, Lambertenghi-Deliliers G. (1986). Immunocytochemistry with back-scattered electrons, in: The Science of Biological Specimen Preparation, M Mueller, R P Becker, A Boyde, J J Wolosewick (eds), SEM Inc AMF O'Hare (Chicago) IL, pp 289-297.

Sternberger LA. (1986). Immunocytochemistry, 3rd ed. J Wiley & Sons, New York.

Studer D, Hermann R. (1986). Colloidal gold particles detected on highly structured surfaces of large samples by backscattered electrons in the scanning electron microscope. in: The Science of Biological Specimen Preparation, M Mueller, R P Becker, A Boyde, J J Wolosewick (eds), SEM Inc, AMF O'Hare (Chicago) IL, pp 203-207.

Takata K, Hirano H. (1984). Whole-cell-mount cytochemistry by the colloidal gold labelling method. Combined transmission and scanning electron microscopic study of Con A binding sites in mouse macrophages. Histochemistry. **81,** 435-439.

Tanaka K. (1981). Demonstration of intracellular structures by high-resolution scanning electron microscopy. Scanning Electron Microsc. 1981; II: 1-8.

Tanaka K, Mitsushima A. (1984). A preparation method for observing intracellular structures by scanning electron microscopy. J. Microscopy. **133,** 213-222.

Trejdosiewicz LK, Smolira MA, Hodges GM, Goodman SL, Livingston DC. (1981). Cell surface distribution of fibronectin in cultures of fibroblasts and bladder derived epithelium. SEM-immunogold localization compared to immunoperoxidase and immunofluorescence. J.Microscopy, **123,** 227-236.

Trejdosiewicz LK, Wilson PD, Hodges GM. (1984). Species cross-reactive membrane-associated urothelial differentiation antigen. J.Nat. Cancer Inst., **72,** 355-366.

Van den Pol AN. (1984). Colloidal gold and biotin-avidin conjugates as ultrastructural markers for neural antigens. Quant.J.Expl. Physiology. **69,** 1-33.

Walther P, Müller M. (1985). Detection of small (5-15 nm) gold-labelled surface antigens by backscattered electrons. Proc. EMSA 1985, San Francisco Press Inc, San Francisco, CA, pp 538-541.

Walther P, Müller M. (1986). Detection of small (5-15 nm) gold-labelled surface antigens using back-scattered antigens, in: The Science of Biological Specimen Preparation, M Mueller, R P Becker, A Boyde, J J Wolosewick (eds), SEM Inc, AMF O'Hare (Chicago), IL 60666, pp 195-201.

Walther P, Ariano B-H, Kriz S, Müller M. (1983). High resolution SEM detection of protein A gold (15nm) marked surface antigens using backscattered electrons. Beitr. elektronenmikroshop. Direktobb Oberfl. **16,** 539-54.

Walther P, Kriz S, Müller M, Ariano BH, Brodbeck U, Ott P, Schweingruber ME. (1984). Detection of protein A gold 15nm marker surface antigens by back-scattered electrons. Scanning Electron Microsc. 1984; III: 1257-1266.

Waterman RE. (1980). Preparation of embyronic tissues for SEM. Scanning Electron Microsc. 1980, II: 21-44.

Wolosewick, JJ. (1984). Cell fine structure and protein antigenicity after polyethylene glycol processing, in: The Science of Biological Specimen Preparation, J-P Revel, T Barnard, G H Haggis (eds), SEM Inc, AMF O'Hare (Chicago), IL 60666, pp. 83-95.

Discussion with Reviewers

M. Horisberger: In this review you describe three-step labelling procedures with colloidal gold. What is your experience with one-step procedures with respect to labelling density and control quality?

Authors: There are several labelling sequences for localization of receptor sites the choice of which is dependent on a number of factors as well-discussed in the literature (cf Sternberger, 1986). Our own labelling procedures for SEM studies have involved in the main two-step and, to a lesser extent, three-step procedures with colloidal gold, both these procedures having been described in this review. The rationale for applying this approach was that the indirect technique allowed a single preparation of gold-labelled secondary antibody to be used for detection of a variety of primary antibodies and that the secondary antibody could provide signal amplification. Nevertheless, in that direct methods may be considered more specific, one-step procedures should be clearly considered.

H Gamliel: Were the non-coated cells treated by non-coating techniques? Most non-coating technique mordants are usually used to intensify the surface osmication of the samples which might also interfere with the BEI image.

Authors: Following immunogold-labelling we have processed specimens for SEM using either secondary osmium tetroxide or OTO schedules followed by carbon coating (cf Figs 6-8; 13-23). Although it has been suggested that post-fixation cannot include osmium tetroxide since the osmium ions would generate a diffuse BEI signal that would obliterate the gold marker signal (de Harven and Soligo 1986) our (albeit limited) experience suggests that osmication may not necessarily interfere with the BEI image.

H Gamliel: It is now recognised that aldehyde fixation followed by osmium treatment is insufficient to protect soft biological specimens from shrinkage and alterations in surface features after critical point drying. The procedure used in the present studies (osmium thiosemicarbazide schedule) might protect the cells from such changes. Is this the reason you processed all the samples by this procedure? Mordant-enhanced osmication was initially introduced for scanning non-coated specimens, why then did you apply the platinum coating? Would not it be better to attempt localizing the marker on the non-coated surfaces?

Authors: Although the rationale for OTO procedures was to obviate the necessity of coating we have found that additional metal coating can help to protect against potential problems of charging and also allow a more detailed study and correlation of specimen morphology and labelled sites using SEI SEM. However, the application of either metal-, carbon- or non-coating procedures is dependent on the information required. In the present studies not all samples were OTO processed, and secondary fixation with osmium tetroxide was also used: additionally, samples were either metal- or carbon-coated with the choice of metal coating in the present studies being aimed at establishing localization and distribution of receptor sites in relation to clearly associated morphological features.

H Gamliel: For pre-fixation different types of fixatives, other than glutaraldehyde, sometimes in 4°C, were used. Did you compare the levels of ultrastructure preservation vs cell-component reactivity under the various conditions applied? What is your recommendation for pre-fixation conditions with regard to minimum effect on target reactivity and maximum preservation of ultrastructure? When do you think we should optimise our fixation -before or after the labelling step? To what extent is it exemplified in your modes of pre- and post-fixation?

Authors: Prefixation has the advantage of preventing post-mortem changes in tissues; in reducing ligand-induced redistribution of cell surface antigens; and can help to anchor cells to substrate particularly following application of cell extraction procedures. Our preference, as exemplified, in these studies, has been to optimise the prefixation step using, in general, paraformaldehyde (PFA). This is a "mild" cross-linking reagent and many surface antigens, for example, will retain antigenicity after PFA prefixation although prolonged fixation can lead to a decrease in labelling intensity. Not all antigens are, however, stable to PFA: for example, many keratin antibodies show poor reactivity with PFA-fixed material. PFA prefixation conditions include the use of a freshly prepared PFA solution; of fixation periods which can range from 20-60 min at ambient temperature for cell cultures through to 2-24h at 4°C for tissues with no gross adverse effects either on ultrastructural or labelling characteristics; and the blocking of reactive aldehyde groups with 0.2M glycine.

D. Soligo: What is your opinion and experience with double-labelling procedures for the SEM?

Authors: To our knowledge only a few SEM studies have applied double-labelling procedures viz: a) primary marking of C. utilis cells with Con A bound to 40nm gold particles and secondary marking with anti-nonmannin antibodies bound to 80nm gold particles (Horisberger et al 1975); b) marking of bovine milk fat globule membranes with WGA-Au_{5nm} and SBA-Au_{18nm} (Horisberger and Rosset 1977b); c) marking of human erythrocytes with SBA-Au_{35nm} and WGA-Au_{75nm} (Horisberger and Rosset 1977b); d) marking of guinea-pig hepatocytes with protein-Au_{40nm} and ferritin-labelled antibodies (Hoyer et al 1979); and e) marking by GAM-Au_{20nm} and GAM-Au_{40nm} respectively of human granulocytes labelled with a mixture of two monoclonal antibodies (one IgG and the other IgM) recognising different surface antigens (Soligo et al 1986). Studies a) to d) were imaged in the SEI mode while study e) showed that gold particles of different sizes could be readily identified in the BEI mode.

Although not yet producing major information double-labelling in SEM studies is a logical extension allowing distinction between two or more distinct but coexisting receptor sites using this microscopic approach.