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DETECTION OF SMALL (5-15nm)
GOLD-LABELLED SURFACE ANTIGENS
USING BACKSCATTERED ELECTRONS

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Abstract

The analysis of surface antigens by scanning electron microscopy represents a practicable alternative to replica techniques. It is generally observed that smaller markers provide for better labelling. In the SEM very small colloidal gold labels however are hardly discerned from contaminants or from surface structures of similar size. They are however unambiguously demonstrated by combining the surface information provided by the secondary electron signal and the material dependent signal provided by the backscattered electrons. A field emission SEM equipped with a highly sensitive single crystal backscattered electron detector allows the routine detection of 5 nm gold particles on biological surfaces. In order to obtain a clear BSE signal of the 5 nm gold particles, the use of material with a high backscattering coefficient (as staining reagent, as coating material or as specimen support) has to be avoided.

Key words Surface labelling, colloidal gold, backscattered electrons, YAG detector, red blood cells, scanning electron microscopy.

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Introduction

In contrast to thin section transmission electron microscopy, scanning electron microscopy permits "three dimensional" analysis of events occurring at the cell surface. Since it is now generally recognized that many important cellular functions are initiated and regulated via interactions taking place at the cell surface, SEM studies of surface antigens have become an area with increasing applications. Surface antigens have been visualized using antibody coupled phages (Kaneshige et al., 1982; de Harven et al., 1979), latex spheres (Quash et al., 1976) ferritin molecules (Hoyer et al., 1979; 1983), colloidal gold (Horisberger and Rosset, 1977; Horisberger et al., 1985; Hoyer et al., 1979) and other markers. These markers are recognized by their specific shape or because of their uniform size.

The colloidal gold marker system has become the most widely used method for the detection of surface antigens. In the TEM colloidal gold marked cell surfaces are usually visualized by replica methods (e.g., Hohenberg et al., this volume). The alternative method in the SEM allows the observation of bulk specimens such as whole cells or tissue. Colloidal gold markers are round particles, which in the secondary electron mode (SE), are hardly discerned from surface structures or contaminants of similar size. Therefore the markers must be amenable to verification using additional information such as X-rays (as proposed by Hoyer et al., 1979) or backscattered electrons (BSE). By combining the surface image provided by the SE and the material dependent BSE-image for the precise detection of the gold markers, an unambiguous localization of surface antigens became possible (Trejdosiewicz et al., 1981; Walther et al., 1983, 1984; Walther and Müller, 1985; de Harven et al., 1984; Molday, 1985; Becker and Geoffroy, 1985).

Large gold particles (15 - 40 nm) can be identified using a conventional SEM equipped with a tungsten filament and a solid state BSE-detector. However experiments (from TEM) show that the rate of labelling increases

when smaller gold particles are used (Slot and Geuze, 1984; de Waele, 1984). This is commonly explained by sterical hinderance (Hoyer, 1979), by lower repulsion forces of small particles (Slot and Geuze, 1984) and by better penetration into the sample (Slot and Geuze, 1984).

The detection of such small particles (5 - 15 nm) in the SEM causes additional problems, because the BSE-signal decreases with the decreasing volume of the gold particles. By using a field emission SEM and a highly sensitive single crystal BSE detector of the YAG type (cerium dotted yttrium aluminum garnet) the detection of 5 nm gold particles becomes possible (Walther and Müller, 1985).

In this paper we present experiments directed to improvements of specimen preparation techniques and signal detection. We shall not deal with the immunological problems.

Materials and Methods

Labelling of red blood cells (RBC)

Fresh heparinized human blood was washed 10 x in PBS (phosphate buffered saline, 300mOsm) at 4°C, fixed with 0.2% glutaraldehyde and 2% paraformaldehyde in PBS, followed by 0.5% Ovalbumin (Sigma) in PBS, washed again three times in PBS and diluted to a concentration of about 3×10^6 cells per ml. One ml of the dilution was incubated with 10 µl of anti-RBC total protein. The incubation was performed at 20°C under constant gentle stirring for 1 hour. After additional intensive washing in PBS/Ovalbumin, the cells were incubated with the different gold solutions: 1) 0.3 ml of the 15 nm protein A gold solution (according to Frens, 1973; coupled according to Roth, 1982) or 2) 10 µl of goat anti rabbit gold (10 nm or 5 nm) (Janssen pharmaceutica). After 1 hour of incubation cells were washed again in PBS, fixed in 0.5% glutaraldehyde for 1 hour at 4°C and finally washed twice with distilled water.

SEM preparation

A glow discharged carbon platelet (3 x 2 x 0.1 mm) (Goodfellow Metals, Cambridge, England) was dipped into the red blood cell suspension and rapidly frozen by plunging into liquid propane (-180°C). The platelets were freeze-dried in a Balzers BAF 300 freeze-etching device. After drying for 1 hour at -80°C the samples were coated with a 10 nm carbon layer by electron gun evaporation. After a final drying step (about 1 hour at -50°C) the samples were warmed up to room temperature and the belljar was vented with dry nitrogen.

The samples were finally mounted on 2 mm carbon rods under an angle of 45° towards the SE detector. These carbon rods fit into a special specimen holder (Nagantani and Okura, 1977). This system allows to apply a variable voltage to the specimen.

Scanning electron microscopy and BSE-detector

A Hitachi field emission SEM S-700 modified for improved resolution as described by Nagantani and Okura (1977) was used. Secondary and backscattered electrons were recorded simultaneously at a measured beam current of approx 5×10^{-11} A and at a working distance between 6 and 8 mm. Secondary electrons were detected by the standard Everhart-Thornley detector. Normally the collection efficiency was optimized by a negative specimen voltage between 0 and -50 volts.

For the detection of the BSE, an annular single-crystal detector (20 mm diameter, central bore 2 mm), placed directly below the objective pole piece, was used. The produced signal was guided to a photomultiplier by a strip of plexiglass which surrounded half of the 2.5 mm thick scintillator disk (Fig. 1). The scintillator disk was glued to the plexiglass using Vitralit 3003 (3M (Switzerland) AG, Zürich) with a refractive index of 1.5. A Hamamatsu R 268 photomultiplier was operated at 750 to 850 V. The used single crystal scintillator was of the YAG type (cerium dotted yttrium-aluminum garnet) described by Autrata (1978), and Autrata et al. (1983). The detector was coated with a conductive layer of aluminum and grounded to the specimen chamber. Special care was taken to coat the small central bore, because charging near the primary beam leads to strong astigmatism.

Results

Fig. 2 shows a red blood cell labelled against total proteins (DAKO A-104). The antibody was visualized by protein A gold (15 nm). After freeze drying the sample was coated with approximately 10 nm of carbon. Occasionally cells, probably cracked during freezing (after the labelling procedure), can be observed. Even at this relatively low magnification, the individual gold particles are visible. The micrograph illustrates that the gold markers on the rear side are not interfering with the signal from the examined face. Otherwise gold signals would also be detected in the cross fractured portion of the red blood cell.

Fig. 3 illustrates a possibility to improve the surface information and to detect the gold particles in an alternative way by applying different voltages to the specimen. For Fig 3A a negative specimen voltage of about -50 V was applied. By this more SE I and SE II can reach the detector (nomenclature of secondary electrons according to Peters, 1982). The surface details are more clearly visible than in the image of the grounded sample (Fig.3C). In Fig. 3D a positive voltage of about +100 V was applied on the specimen. Thus no SE originating from the specimen can reach the detector. The signal is produced by backscattered electrons which are converted into SE III at the walls of the

Discussion

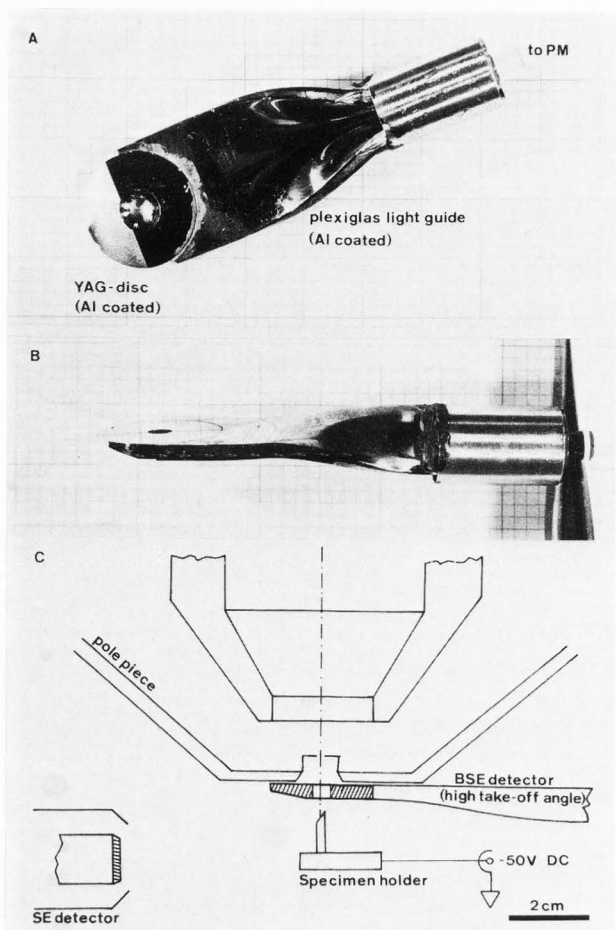


Fig. 1: The YAG detector for backscattered electrons.

Fig. 1A and 1B shows the YAG disc and the plexiglas light guide to the photomultiplier (PM).

Fig. 1C shows the situation in the specimen chamber: The detector, positioned below the pole piece, collects the BSE with a large take-off angle. In order to optimize the collection efficiency of the SE I and SE II, a negative voltage can be applied onto the specimen.

specimen chamber, the YAG-detector and the pole piece. This signal is very similar to the BSE-image, detected with the YAG-detector (Fig. 3B).

Fig. 4: At sufficiently high primary magnifications even individual 5 nm gold particles are detected with the procedure and instrumentation outlined above. This red blood cell is labelled as described in Fig. 2 except that the antibody was visualized with a second antibody coupled to 5 nm colloidal gold. Fig. 4A shows the SE image, Fig 4B the corresponding BSE image. Fig. 4C and 4D show corresponding details of Fig 4A and 4B. In Fig. 4D the individual 5 nm gold particles are visible.

For the detection of small gold particles on biological surfaces by backscattered electrons the following points should be taken into account:

The use of heavy metals for fixation or as specimen supports should be avoided.

An acceptable image of the surface is obtained only when the specimen is electrically conductive. This conductivity, however, must not be produced by chemicals or coating materials with high backscattering coefficients. Otherwise the BSE signal of very small gold particles gets obscured. To enhance the conductivity we use carbon coating. For small specimens (e.g., single cells) a layer of 5 to 10 nm is sufficient. When large specimens such as tissue pieces are used, charging becomes a serious problem, which is discussed in the paper of Studer and Hermann (this volume). By the presently available labelling techniques using protein A gold or gold labelled second antibodies, an antigen can be localized with an accuracy of 20 to 30 nm. Compared to this the surface information obtained with the relatively thick carbon layers may be appropriate. It may be improved by the use of very thin continuous coating layers of materials with low backscattering coefficient (Peters, 1985). Such layers are, however, not easily obtained.

In order to minimize the noise contribution of the specimen support, carbon platelets proved to be very useful. Their backscattering coefficient is low and they are electrically conductive. The platelets however, are easily deformed. Therefore, careful handling during freeze preparation is necessary.

As demonstrated in Fig 3D, colloidal gold particles can also be detected by the BSE converted into SE III when a positive voltage is applied to the specimen. (BSE detectors collecting an enhanced SE III signal are proposed by Moll et al., 1978; Robinson, 1979 and by Reimer and Volbert, 1979.) Labelling experiments are, however, greatly facilitated by the use of a BSE detector because both, the SE and the BSE image can be observed simultaneously. In addition, the application of a voltage to the specimen changes focus and specimen position.

The presented approach to localize gold labelled surface antigens by combining the surface information provided by the SE signal and the identification of the gold markers by the material dependent BSE signal represents a powerful alternative to surface labelling using replica techniques (Hohenberg et al., this volume). If a field emission SEM equipped with a highly sensitive BSE detector is used, 5 nm gold markers are routinely detected. Thus an information density similar to transmission electron microscopy is obtained. For both SEM and TEM, however, numerous problems associated with the maintenance of the structural and functional integrity remain to be solved.

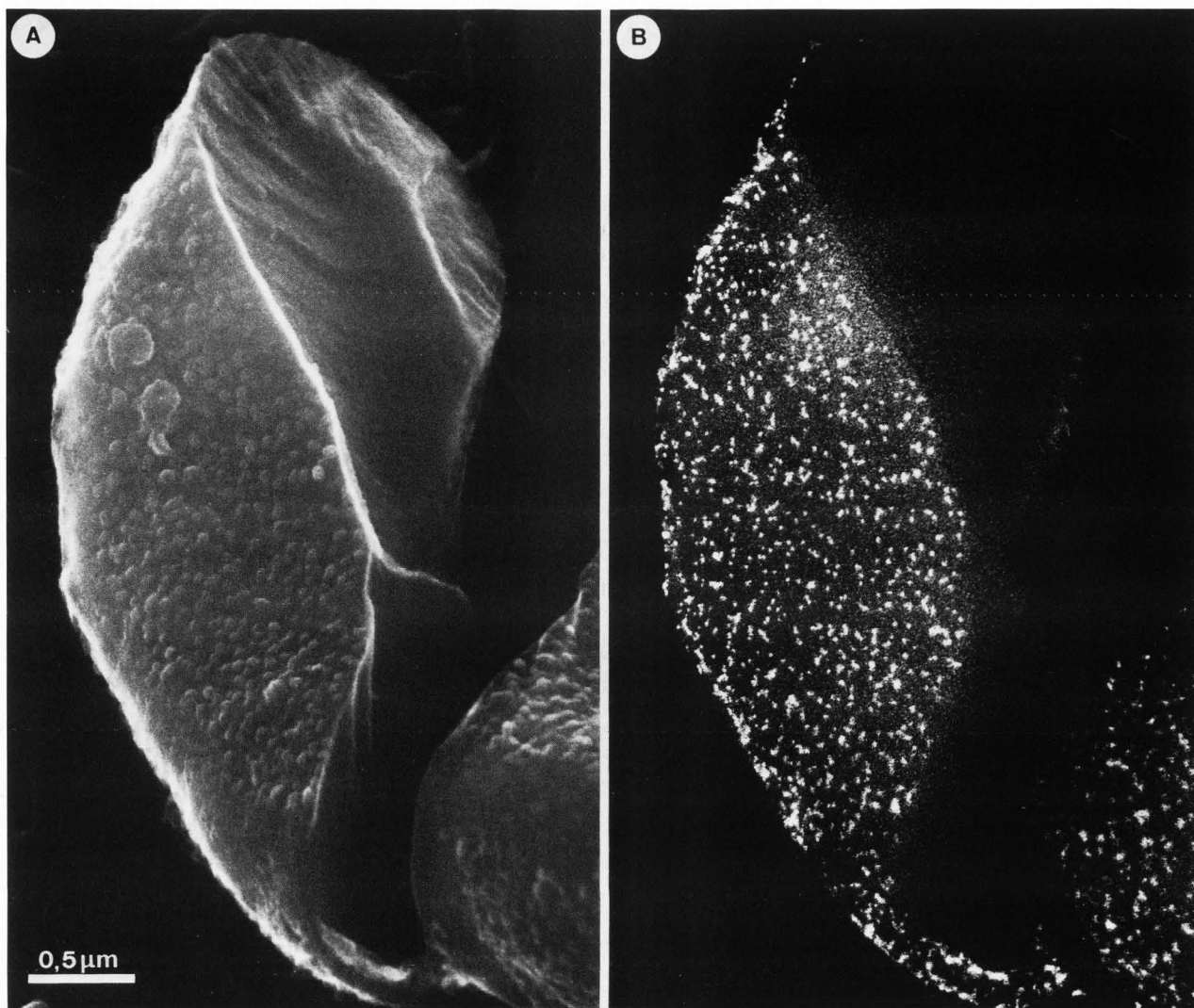


Fig. 2: A red blood cell, labelled against the total protein with an antibody and protein A gold (15 nm). The cell is cross-fractured (probably during freezing). Fig. 2A: Secondary electron mode, Fig. 2B: Backscattered electron mode.

Even at this relatively low magnification, the individual 15 nm gold particles are visible. The gold markers at the rear of the RBC are not interfering with the signal, otherwise, one would see them in the fractioned region.

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BSE Image of Colloidal Gold

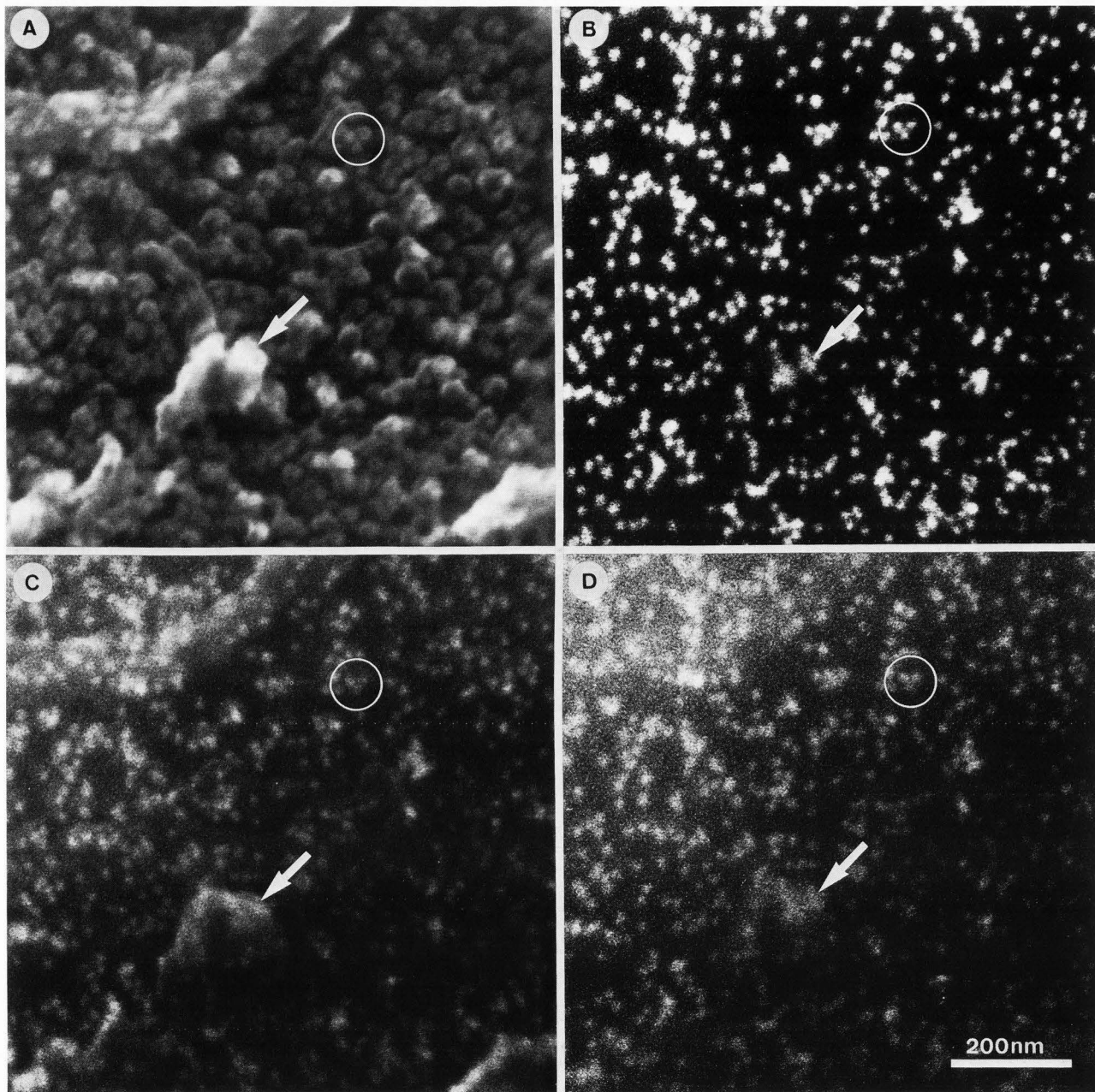


Fig. 3: A portion of a red blood cell, labelled against the total protein with an antibody and protein A gold (15 nm). The figure demonstrates the influence of a voltage applied onto the specimen. Fig. 3C: SE image without an additional specimen voltage. Fig. 3A: SE image with a negative specimen voltage of about -50 volts. The SE I and SE II signal is enhanced and the surface structure is more clearly visible than in fig. 3C (arrow). Fig. 3D: SE image with a positive specimen voltage of about 100 volts. Now the SE from the specimen (SE I and SE II) cannot reach the SE detector and the signal is produced by BSE converted into SE III at the specimen chamber. The image becomes similar to the image collected with the BSE detector (Fig. 3B).

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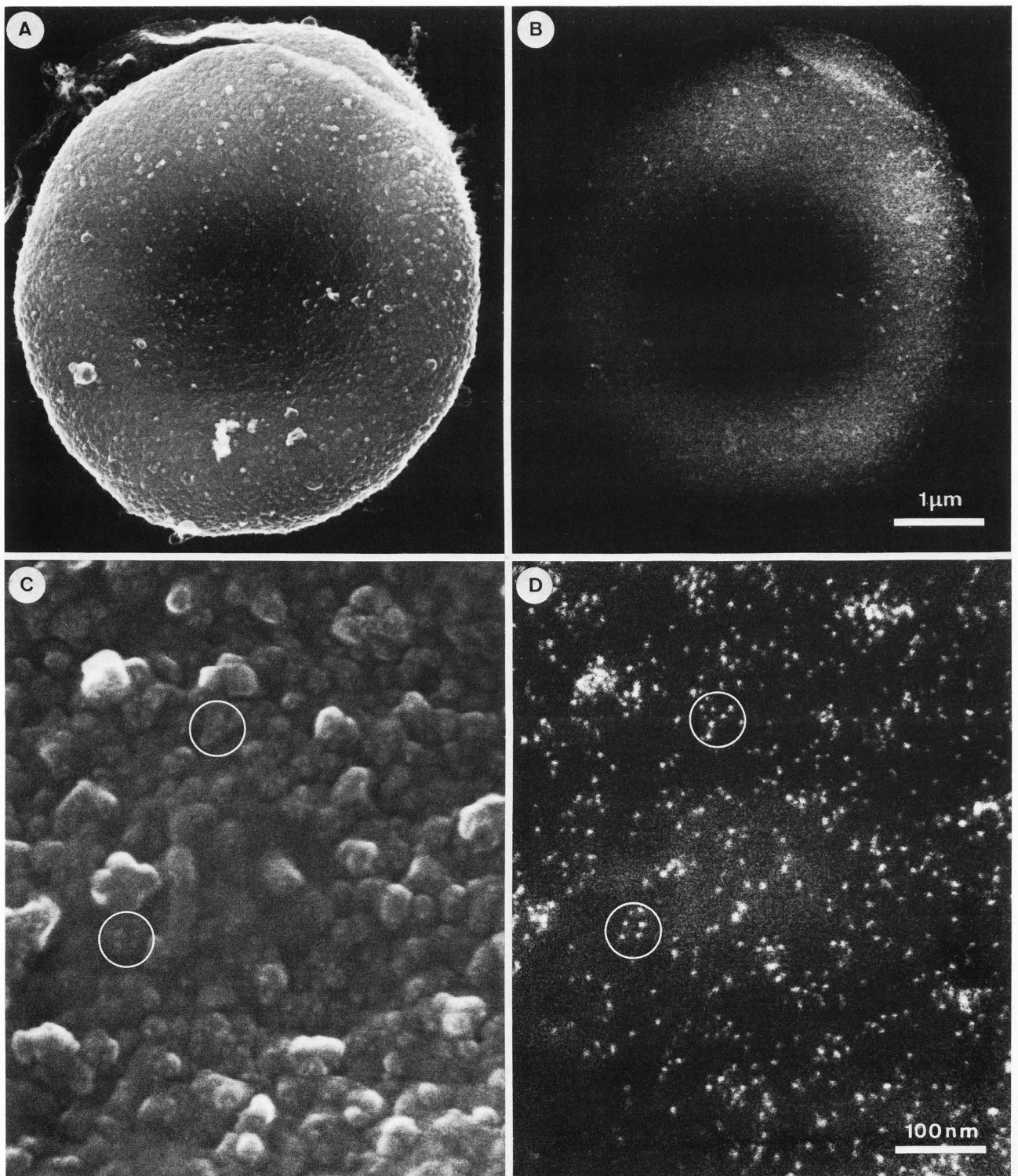


Fig. 4: Red blood cell labelled with antibodies against the total protein and with a second antibody coupled to 5 nm colloidal gold. Fig. 4A: SE image, Fig. 4B: BSE image. Fig. 4C and 4D: A portion of the same cell at higher magnification.

At high magnification the individual 5 nm gold particles are visible on the cell surface in the BSE mode (Fig. 4D). Circles denote same area in the SE and in the BSE image.

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