Immunohistochemical localization of cell surface receptors using a novel method permitting simple, rapid and reliable LM/EM correlation

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Summary
A method is presented which allows correlative serial section analysis by light and electron microscopy of cell surface antigens in monolayer cultures. Sites of antigenicity are shown by deposition of diaminobenzidine after pre-embedding, immunoperoxidase immunocytochemistry. Osmication is replaced by the use of gold chloride which specifically enhances the electron density of diaminobenzidine. In addition gold chloride bound to diaminobenzidine survives embedding and provides the basis for a post-embedding photochemical amplification method. Immunostained cells are embedded in LR White by a rapid technique which preserves their structure and leaves them available for subsequent post-embedding immunocytochemistry. The method is illustrated by the demonstration of epidermal growth factor (EGF) receptors on the EGF receptor-rich human carcinoma cell line A431 using a well characterized monoclonal antibody raised against EGF receptor.

Introduction
High resolution cytochemical localization of cell surface membrane receptors has recently been achieved most effectively for epidermal growth factor (EGF) receptors using both ligand histochemical (EGF-ferritin or EGF horse radish peroxidase conjugates) and immunocytochemical (EGF-R1, an EGF receptor specific monoclonal antibody) probes (Willingham et al., 1983; Beguinot et al., 1984). These studies, however, have mainly been directed towards the examination and elucidation of the topographical distribution and behaviour of the EGF receptors on an individual cell basis, with little or no consideration given to the question of cell to cell variation in EGF receptor numbers and distribution. There is, however, strong evidence from flow cytometric studies to suggest that growth factor specific receptor numbers even in a genetically homogeneous population of a single cell type can vary widely between individual cells (Cantrell & Smith, 1984).

In order to accommodate study of such differences in an otherwise high resolution immunocytochemical analysis, it has been found necessary to devise a new pre-embedding–post embedding system which affords a high sensitivity LM visualization of cell membrane receptors present on many cells included in a semi-thin section, but at the same time allows the detailed EM examination of the receptor status of selected individual cells in an adjacent ultra-thin section. Particulate markers such as colloidal gold and ferritin have been preferred by some workers for pre-embedding immunoelectron microscopy owing to the unequivocal character of the markers. In particular a four-stage procedure based on ferritin has proved very successful in the analysis of growth factor receptors (Beguinot et al., 1984; Willingham & Pastan, 1985). The major limitation of this technique is that LM immunostaining cannot be achieved on the same cell preparation. Colloidal gold, however, although often only weakly visible, has been shown to have the potential for photochemical enhancement and so may provide a basis for LM/EM studies. (For a review see Lucocq & Roth, 1985). In order to facilitate this, post osmification would of course have to be avoided (or used after intensification) and the sensitivity of such a method as applied to surface receptors is unknown. It has also been implied that colloidal gold may not be an appropriate marker in view of the general propensity of cells to endocytose it, which could be confused with receptor internalization (Willingham et al., 1983).

In the present study the application of our new approach is described in detail with respect to the localization of the EGF receptor associated with the A431 human carcinoma cell line (Fabricant et al., 1977).

Materials and methods
CELL CULTURE
A431 cells were plated on 13 mm plastic tissue culture coverslips
IMMUNOSTAINING

Coverslips were briefly rinsed of medium in phosphate buffered saline (PBS), pH 7.4, and then fixed for 10 min at room temperature in 3% glutaraldehyde in 0.1 M phosphate buffer. Before immunostaining the cells were treated with phenylhydrazine and hydrogen peroxide (to inhibit endogenous peroxidase). For this, coverslips were immersed in 50 μM phenylhydrazine solution in PBS at 37°C for 10 min, following which H₂O₂ was added to 0.0003% and the incubation continued for a further 20 min (Jasani et al., 1986). After washing (3 × 2 min in PBS) the coverslips were covered with 30 μl of primary antibody solution. The anti-EGF receptor antibody was the mouse monoclonal EGF-R1 antibody generously supplied by Dr M. D. Waterfield (ICRF, London). The specificity of this antibody for the EGF receptor has been demonstrated by its ability specifically to precipitate a 175 kD cell surface polypeptide which binds [125I]-labelled EGF and possesses EGF stimulable protein kinase activity (Waterfield et al., 1982). It was diluted to approximately 2 μg/ml in a freshly made 0.1% solution of bovine serum albumin in PBS (BSA-PBS). The control antibody used was a mouse monoclonal antibody to bromo-deoxyuridine (Impex Laboratory, Twickenham, UK) used at the same concentration. After incubation with the primary antibody for 45 min at 37°C in a humidified chamber, the coverslips were washed in PBS (3 × 2 min) and then covered with 30 μl of the second antibody reagent: rabbit anti-mouse immunoglobulin conjugated to horse radish peroxidase (Dakopatts) at a dilution of 1:100 in BSA-PBS. After 30 min incubation at room temperature, the coverslips were again washed in PBS and taken through a final incubation for 5 min in 0.05% diaminobenzidine–0.005% hydrogen peroxide in PBS for the development of the peroxidase reaction. Immunostained coverslips were then either mounted in Canada balsam (after dehydration and clearing) for light microscope viewing of whole cells or processed for sectioning as below.

PROCESSING

Whole coverslips containing experimental and control cells were thoroughly washed (3 × 5 min) in distilled water in a watchglass before being immersed in either 25 μM gold sodium chloride, Na₂AuCl₄·2H₂O (BDH, Poole, UK) dissolved in distilled water or a solution of 2% osmium tetroxide in veronal acetate buffer for 15 min. They were again washed as above, then dehydrated in a graded acetone series (5 min each in 50%, 70% and 100%) and cleared in chloroform. To avoid problems with evaporation, this stage was best completed with the coverslips upside down, two changes of 5 min each being quite sufficient. After clearing, the gold chloride treated coverslips were placed in pure unpolymerized LR White and the osmicated ones into pure Epon 812. The coverslips were then turned the right way up and given three 5 min changes of LR White or Epon as appropriate. Before final LR White or Epon embedding the coverslips were cut up with scissors usually into 6 or 8 parts each, taking care to keep them with the cells on the upper surface.

LR White embedding

LR White was mixed with accelerator (as supplied by the manufacturers) at room temperature, in aliquots of 10 ml plastic to which 15 μl of the accelerator were added, and thoroughly stirred for 30 s (Yoshimura et al., 1986). Provided that both the plastic and the accelerator were not over 3 months old (prepolymerized) and had been stored in a refrigerator at 4°C there were at least seven minutes available during which to complete the remaining embedding manoeuvres before the plastic began to gel. Flat-bottomed polypropylene ‘Beem’-type capsules with their lids removed were quickly filled with the plastic accelerator mixture. A piece of coverslip was sited with its cells uppermost on the bottom of a foil flat-embedding dish and an LR White filled capsule was inverted over it. This process was repeated until all the pieces of coverslip were embedded. Residual LR White plastic was poured around the base of the inverted capsules to take up the space caused by polymerization shrinkage. The plastic polymerized within 45 min, by which time it was possible to simply pull off the blocks from the foil making them immediately available for shaping up and removal of the coverslip pieces. A slight rise in temperature occurred during polymerization in the capsules, but the small volume prevented it from being any greater than 50°C. Some unpolymerized monomer always remained in the end of the capsules giving the blocks a ‘wet’ end distal to the tissue.

When the excess plastic was cut or filed away the coverslip could be gently levered away with the corner of a razor blade leaving the intact cells embedded in the face of the block. The face was then reduced to a size compatible with semithin and ultrathin sectioning. In some cases the ‘face’ was cut off from the front of the capsule and re-embedded at right angles to facilitate the sectioning of the cells perpendicular to the plane of the monolayer.

Epon 812 embedding

‘Beem’-type capsules filled with Epon were inverted over pieces of coverslip on foil flat embedding dishes in a similar way to that described for LR White. However polymerization was conducted at 65°C overnight.

Serial semithin and ultrathin sections were cut using glass knives on a Reichert Ultratome ‘Autocut E’. Up to 12 serial semithin sections could be cut from a block prepared from one piece of coverslip provided that the sections did not exceed 350 nm in thickness. A routine was adopted in which six serial semithin sections were cut onto a water-boat, collected and dried down onto numbered chrome-gel-coated glass slides at 50°C for one h, and six serial ultrathin sections (less than 90 nm thick) were cut and collected on formvar-coated, carbon-coated copper slot-grids with a 2 × 1 mm slot (Agar Aids Ltd, Stansted, UK). In this way all the cells cut could be seen in the EM without the interference of grid bars and direct comparison could be made with cells shown in serial semithin sections.

LM EXAMINATION

Semithin sections of Epon and LR White embedded tissue were either directly mounted under coverslips in Gurr’s neutral mounting medium for viewing in the light microscope or photochemically enhanced using a silver amplification method before mounting.