# Label-Fracture: A Method for High Resolution Labeling of Cell Surfaces

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ABSTRACT We introduce here a technique, "label-fracture," that allows the observation of the distribution of a cytochemical label on a cell surface. Cell surfaces labeled with an electron-dense marker (colloidal gold) are freeze-fractured and the fracture faces are replicated by plantinum/carbon evaporation. The exoplasmic halves of the membrane, apparently stabilized by the deposition of the Pt/C replica, are washed in distilled water. The new method reveals the surface distribution of the label coincident with the Pt/C replica of the exoplasmic fracture face.

Initial applications indicate high resolution (≤15 nm) and exceedingly low background. "Label-fracture" provides extensive views of the distribution of the label on membrane surfaces while preserving cell shape and relating to the freeze-fracture morphology of exoplasmic fracture faces. The regionalization of wheat germ agglutinin receptors on the plasma membranes of boar sperm cells is illustrated. The method and the interpretation of its results are straightforward. Label-fracture is appropriate for routine use as a surface labeling technique.

The localization of cell-surface receptors, antigens, and other chemical groups associated with lipid or protein molecules of the plasma membrane is a recognized objective of the cell sciences. Ultrastructural cytochemistry of cell surfaces was first observed in thin sections of cells and tissues, a process that required the assemblage of composites obtained from serial-sectioned cells (1). To overcome this unusually difficult procedure, other techniques were developed that allowed direct views of the surface distribution of the label: (*a*) plasma membranes collapsed at an air/water interface (2, 3); (*b*) platinum/carbon replication of cells (air-dried, freeze-dried, or critical-point dried) (4); and (*c*) platinum/carbon replication of frozen cells after freeze-fracture and sublimation (freeze-etching) (5–9). All these approaches suffered from severe limitations. Collection of unfixed (or lightly fixed) membranes at the air/water interface could not prevent reorganization of receptors (10). Conventional Pt/C replication of dried specimens could only be applied to cell monolayers (or to cells previously attached to a substrate), and drying procedures could cause deformation and collapse of structures (11, 12). Freeze-etching related the surface distribution of receptors and antigens to that of the intramembrane particles revealed by freeze-fracture but could only be performed in systems (generally isolated membranes) capable of withstanding freezing in the absence of cryoprotectants.

Over the past few years, we have developed new labeling techniques—fracture-label (13–16)—that allow direct, in situ labeling of freeze-fractured plasma and intracellular membranes. As freeze-fracture splits biomembranes along their bilayered continuum (17, 18), fracture-label techniques can be used to determine the sidedness of membrane components and, in some instances, to identify transmembrane proteins as well as their regionalization along the plane of the membrane (19–23). Because labeling is done after freeze-fracture, fracture-label was not intended as a substitute for surface labeling. Moreover, thawing of freeze-fractured specimens (required for labeling of freeze-fractured membranes) led to postfracture reorganization of membrane components (13, 15, 20). This resulted in ultrastructural alterations that made impossible a direct correlation of the distribution of the label to that of the intramembrane particles seen in conventionally freeze-fractured specimens.

We describe here a simple technique that allows the visualization at high resolution of cytochemical markers of cell-surface sites over large cell-surface areas. This distribution is seen superimposed on the unaltered image of a freeze-fractured exoplasmic fracture face (E face)[1] (24). In brief, cell-surface receptors are labeled with colloidal gold techniques and freeze-fractured. Contrary to conventional freeze-fracture, Pt/C-replicated specimens are not digested with acids or

[1] *Abbreviations used in this paper:* Con A, concanavalin A; E face and P face, exoplasmic and protoplasmic fracture faces, respectively; WGA, wheat germ agglutinin.

bases. Instead, they are repeatedly washed in distilled water. The labeled outer half of the membrane remains attached to the Pt/C replica. This allows the simultaneous observation of the surface label and the Pt/C replica of the exoplasmic half of the plasma membrane in one single, coincident image.

## MATERIALS AND METHODS

*Cells:* Human peripheral blood (B and O, positive) was washed three times in PBS. Cell suspensions enriched in leukocytes were obtained by discarding about two-thirds of the erythrocytes. The cells were then fixed in 1.5% glutaraldehyde in PBS, pH 7.4, for 1 h at 4°C and washed twice in PBS before labeling. A suspension of thymic cells from the pooled thymi of six mice (C3H, 10-wk old) was prepared (25) and the cells were incubated at 37°C for 30 min and fixed as above. Samples of sperm from mature boars, *Sus scrofa* (a gift of Dr. Larry Johnson of the U. S. Department of Agriculture, Beltsville, MD), were washed three times in PBS or in Hank's balanced salt solution (19), fixed, and washed as above.

*Surface Labeling:* Samples of ~0.1 ml of packed cells were used. For wheat germ agglutinin (WGA) labeling, cells were resuspended in PBS and incubated in a solution of 0.25 mg/ml WGA in 1 ml PBS for 1 h at 37°C. Controls were preincubated in 0.4 M *N*-acetyl-D-glucosamine for 15 min at 37°C followed by incubation in a WGA solution as above but in the presence of 0.4 M *N*-acetyl-D-glucosamine. After being washed twice in PBS, the cells were incubated in ovomucoid-coated colloidal gold (26, 27) for 3 h at room temperature. For concanavalin A (Con A) labeling, 0.1 ml of packed cells was resuspended in PBS and then incubated in a solution of 0.25 mg/ml Con A in 1 ml PBS for 1 h at room temperature. Controls were preincubated in 0.5 M methyl-α-D-mannopyranoside for 15 min at room temperature followed by incubation in a Con A solution as above in the presence of 0.5 M methyl-α-D-mannopyranoside. The cells were washed twice in PBS and labeled by incubation in a suspension of horseradish peroxidase-coated colloidal gold solution (26, 27) for 3 h at room temperature.

*Freeze-Fracture:* Fixed, labeled cells were impregnated in 30% glycerol, mounted on double-replica copper disks, frozen, freeze-fractured at −130°C, and replicated by Pt/C evaporation. Instead of the conventional cleaning of replicas in acids or bases, replicas were washed by at least six successive floatings on distilled water (30 min per wash). After washing, the replicas were mounted on Formvar-coated grids for electron microscopic examination. Complementary replicas were produced with a double-replica device. While one of the replicas was washed in distilled water as above, the other was digested in sodium hypochlorite before washing.

## RESULTS

The experimental sequence of label-fracture is schematically illustrated in Fig. 1. We first observed human erythrocytes that had been treated with Con A (Fig. 2) or with WGA (Figs. 3 and 4) and then labeled by colloidal gold coated with horseradish peroxidase or with ovomucoid, respectively. Fig. 2 shows that the label is observed superimposed with the conventional, high-resolution image of the E face. This label remains in place after the repeated washing needed to remove unfractured, unreplicated cells in suspension. As the colloidal gold label is *under* the fracture face (i.e., is attached to the *outer surface* of the outer, or exoplasmic, half of the membrane; see Fig. 1, *a* and *b*), it is not replicated. Therefore, it does not produce cones of shadow. This happens only with colloidal gold particles at the edge of the fracture face, where the outer half of the membrane is cross-fractured. Here, shadowed colloidal gold particles frequently line the edge of the E face generally at a distance of 5–10 nm (Fig. 4; see also Fig. 1). Controls—i.e., human erythrocytes and leukocytes incubated with lectin in the presence of competing sugars—showed drastic reduction of labeling intensity (80% or more) and demonstrated specificity (Fig. 5).

Observation of the E faces of labeled leukocytes also showed specific labeling against a clean background (Fig. 6). Colloidal gold particles were confined to within 10–20 nm of the fracture face.

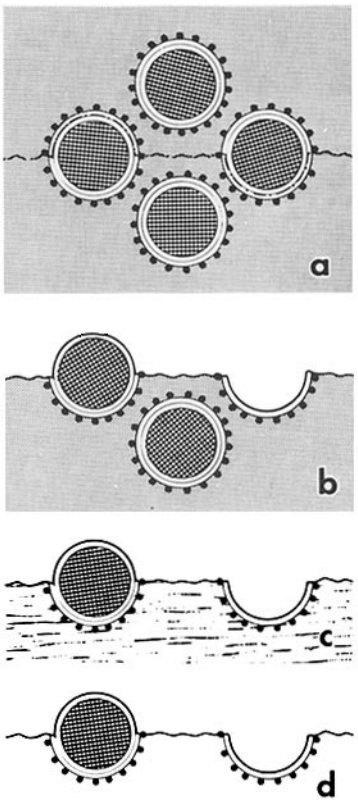
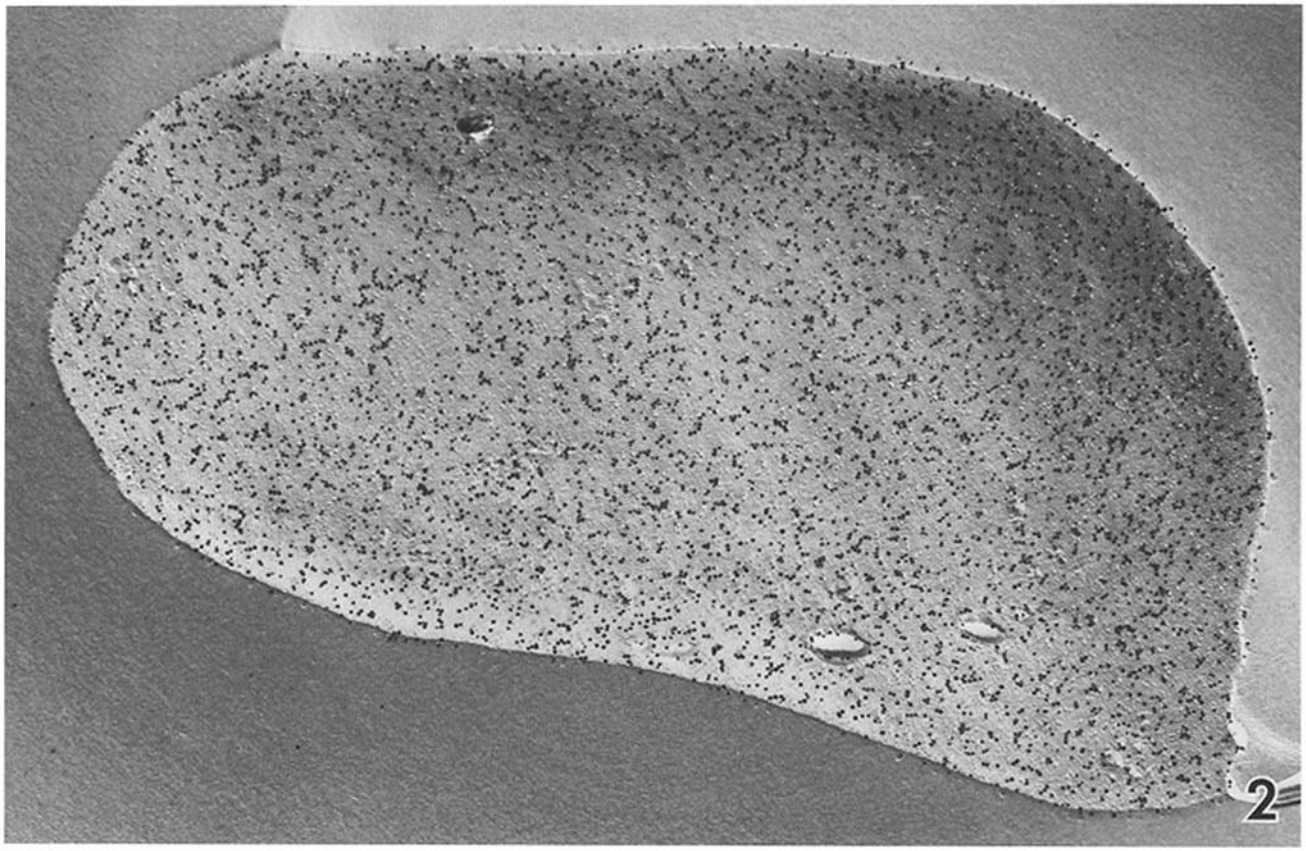


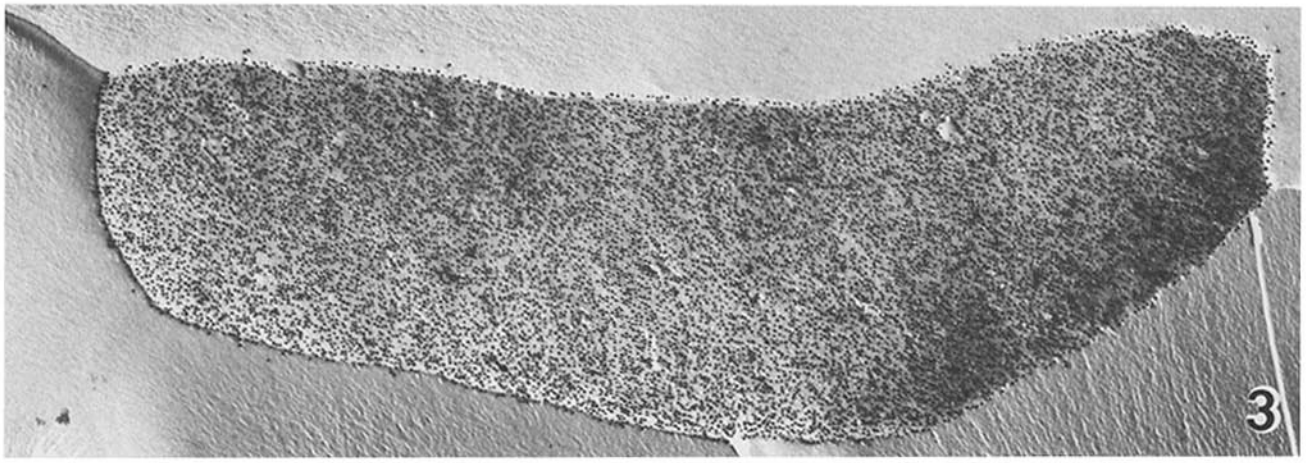

FIGURE 1 Label-fracture. (*a*) Cells in suspension are labeled and frozen. (*b*) Freeze-fracture splits the plasma membranes of labeled cells into exoplasmic halves (with attached surface label; *right*) and protoplasmic halves (which remain attached to the cell body; *left*). Pt/C evaporation produces a high-resolution cast of the fractured cells (only label at the interface of fracture is exposed and shadowed; see also Fig. 4). (*c*) Fractured, shadowed specimens are thawed and washed with distilled water which removes unfractured cells. Exoplasmic membrane halves remain attached to the replica. Coincident images of the Pt/C replica of the E face and the surface label are produced. Cells with fractured P faces (*left*) remain attached to the replica (here, electron density of the cell body prevents observation of the P face). (*d*) The replicas are then mounted on electron microscope grids, dried, and observed.

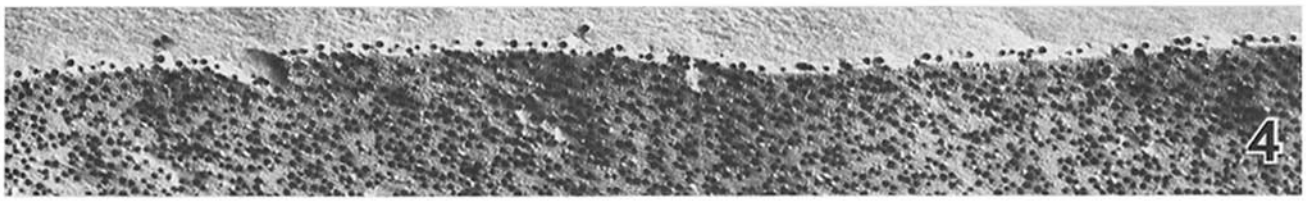
Cross fractures of cell projections and invaginations in lymphocytes showed two distinct patterns of labeling: (*a*) cross-fractures of projections were generally labeled with the labeling frequently obscured owing to the electron density of the undigested, cross-fractured cytoplasm (Fig. 6, white arrows and inset); (*b*) cross-fractured invaginations (less frequent) were clean and, in general, labeled only at the perimeter (Fig. 6, black arrows and inset).

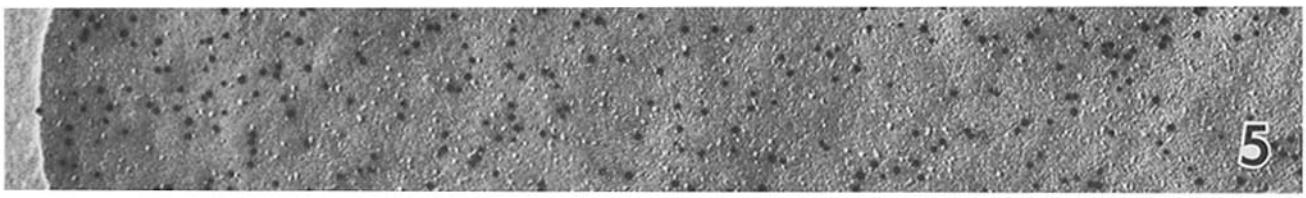
High resolution of the labeling was also demonstrated by the observation of the co-distribution of the label with aggregates of intramembrane particles[2] induced in cells from thymic suspensions by incubation at 37°C for 30 min (28). In those cells (Fig. 7), colloidal gold particles were almost exclusively confined to the particle aggregates leaving the intervening areas (rich in small rugosities) unlabeled: Fig. 7 shows that of 588 gold particles over the E face, only two are not confined to within 10–15 nm of the particle aggregates. This confirmed

[2] Aggregation of integral membrane proteins in human erythrocyte ghosts at pH 5.5 was also attempted. The experiments failed because the massive, irreversible agglutination by the lectin prevented susequent labeling with colloidal gold.

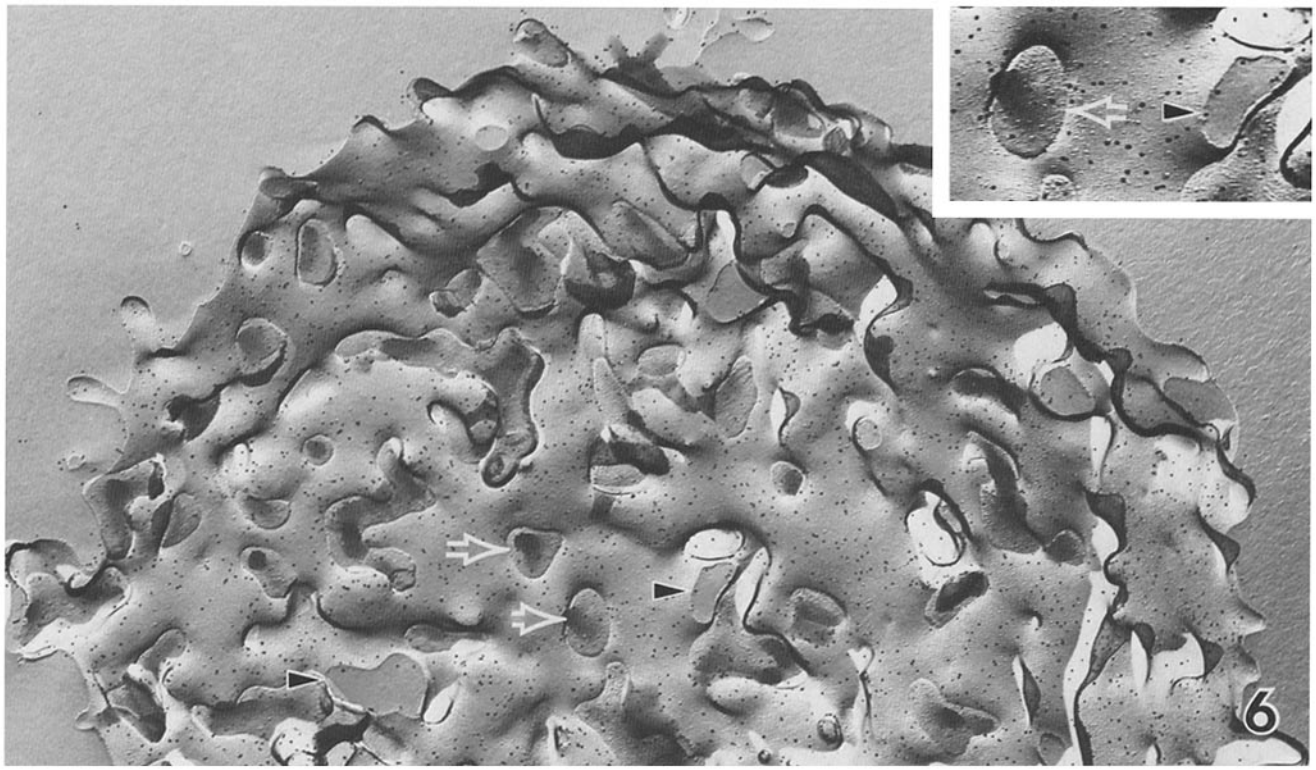


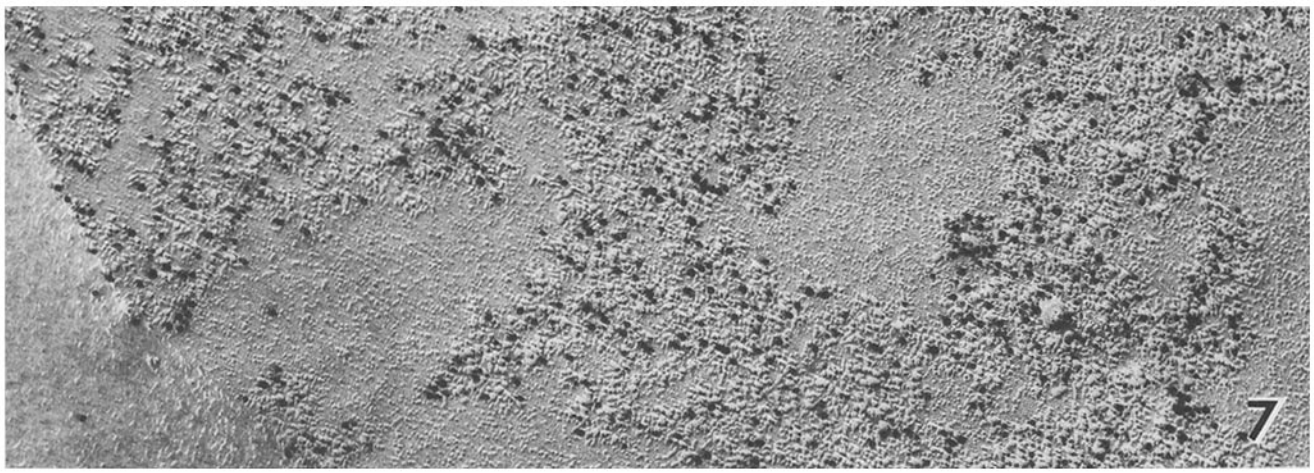




FIGURES 2–5 Label-fracture of human erythrocyte membranes. Fig. 2: Con A; Figs. 3–5: WGA. (Fig. 5: Control; WGA labeling in the presence of 0.4 M *N*-acetylglucosamine.) In all cases, label is confined to within 15 nm of the edge of the exoplasmic face. Colloidal gold particles at the edge of the fracture face are frequently shadowed (see Fig. 4; see also Fig. 1, *b* and *c*). For WGA, average density of label is 1,484 ± 56 colloidal gold particles/$\mu m^2$ (Figs. 3 and 4); density in control preparation (Fig. 5) is 253 ± 19/$\mu m^2$ (labeling reduction: 83%). Fig. 2, × 45,000; Fig. 3, × 30,000; Figs. 4 and 5, × 62,000.




FIGURES 6 and 7 Fig. 6: WGA label-fracture of a human leukocyte. Label is confined to 10–20 nm of the fracture face. During freeze-fracture, cell projections and invaginations are cross-fractured (see Results for interpretation of labeling). × 30,000; (*inset*) × 50,000. Fig. 7: When thymocyte cell suspensions are incubated in PBS at 37°C, intramembrane particles in many cells aggregate. This is used here to illustrate the resolution of label-fracture: Con A-binding sites in this cell are restricted to the particle aggregates (outlined): of 588 gold particles, only two are not associated to the aggregates. × 62,000.

the resolution of the labeling (≤15 nm).

Label-fracture of boar sperm cells (Fig. 8) illustrated the capacity of the new technique to define the regional distribution of a surface receptor. WGA binding sites were denser over the neck and midpiece of the tail. Over the postacrosomal area of the sperm head plasma membrane, the density of receptors was decreased with the exception of the rim that surrounds the base of the head. The indentations of sperm neck cords at the base of the head were not labeled (Fig. 8). The distribution of label was not related to that of the intramembrane particles shown on E faces.

To explore the possibility of directly relating the label on E faces to the freeze-fracture morphology of protoplasmic fracture (P) faces, we searched for complementary aspects in paired replicas, one of which was digested in sodium hypochlorite. In Fig. 9 we show complementary aspects of P and E faces of a label-fractured erythrocyte. Surprisingly, the P face (Fig. 9, left) is clean of contaminating colloidal gold particles which are clumped and confined to the surrounding medium. The reason for this fortunate result is the following: during thawing and cleaning of the replicas, P faces remain sheltered from colloidal gold contamination by the glutaraldehyde-fixed cell underneath. Digestion by sodium hypochlorite followed by washing in distilled water yields clean P faces.

## DISCUSSION

We introduce here a method "label-fracture," for the labeling of cell surfaces in images superimposed onto, in fact, coincident with, those of high resolution Pt/C replicas of exoplasmic fracture faces. The method appears to have wide application

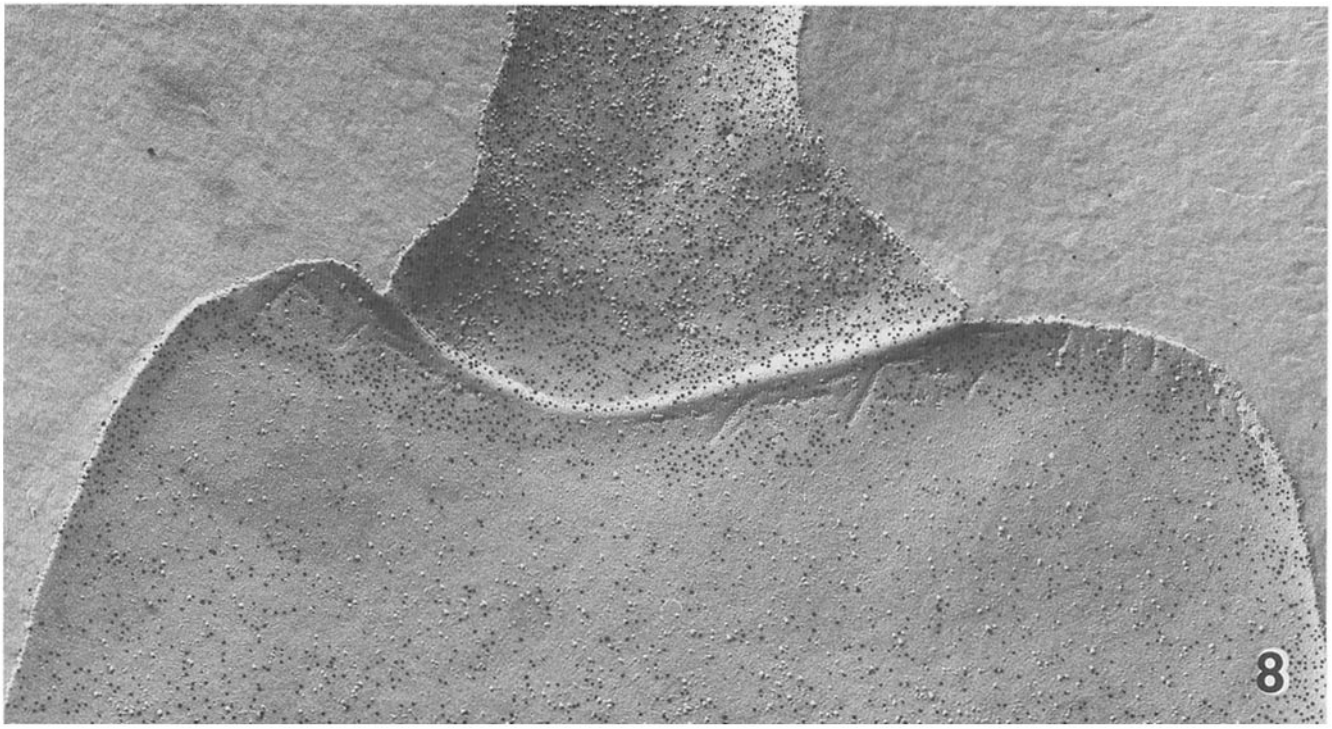



FIGURE 8 Label-fracture reveals the regionalization of WGA receptors on the plasma membrane of boar sperm. See Results for description. × 52,000.

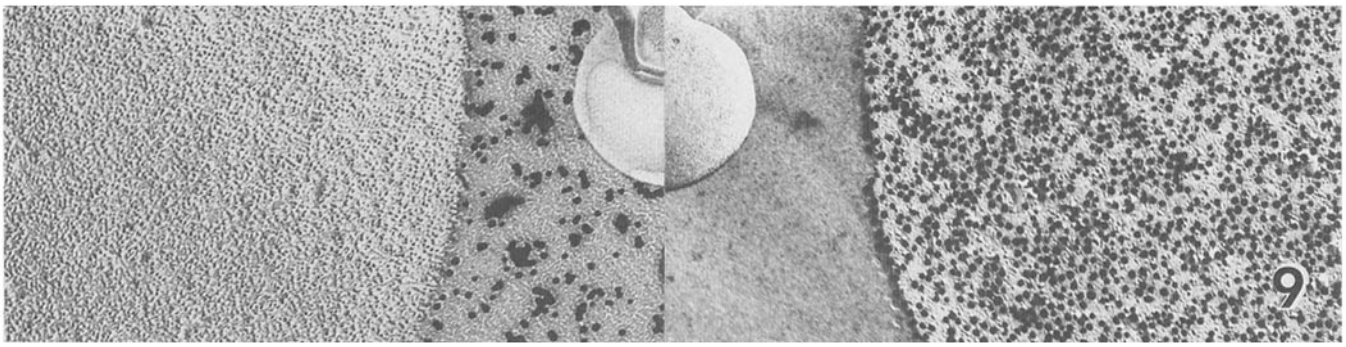



FIGURE 9 Complementary views of a WGA label-fractured human erythrocyte (see Results for interpretation). × 62,000.

and is technically simple. The results are easy to interpret. While we chose WGA and Con A to illustrate the potential of label-fracture, antibodies coupled to colloidal gold or labeled by colloidal gold coated with protein A can be used to locate other surface receptors and antigens.

The most remarkable and crucial feature of this method is the retention of the label at the surface of exoplasmic membrane halves after freeze-fracture and washing in distilled water. This may seem suprising at first, but it is easily explained. After fracture, the split outer half of the plasma membrane is coated with a stabilizing layer of Pt/C. After fracture and replication, when the frozen specimens are thawed and the replicas are floated in distilled water, all unfractured cells are washed off. Repeated washing assures clean replicas with minimal background label. Our results indicate that this process retains and does not structurally alter freeze-fractured exoplasmic membrane halves. This is to be expected: thermodynamically freeze-fractured exoplasmic halves of the plasma membrane are stable, monolayered structures with their polar (outer) surface facing water and their apolar face turned upwards, where it is stabilized by a Pt/C (~2-nm thick) replica reinforced with a layer of carbon (~10–20-nm thick). Therefore, there is no reason to suspect a postfrature reorganization into bilayered structures of the type we have shown to be produced when freeze-fractured membranes are thawed directly in aqueous solutions. (See references 13, 15, and 20 for discussion.) Future studies will determine whether colloidal gold labeling of transmembrane proteins that preferentially partition with the protoplasmic half of the membrane remains attached at the outer surface of the membrne or is lost during washing. Our preliminary experiments indicate that thawing of the fractured specimens in glutaraldehyde solutions does not result in denser labeling.[3]

[3] Rash et al. (29) proposed a method ("labeled-replica") for labeling of platinum-shadowed freeze-fractured specimens. This method appears to be at an incipient phase of development. We have found it impossible to correlate the description of the results obtained with these methods with the few micrographs published (see Figs. 4–6 in reference 29). In "labeled-replica" preparations, fractured faces are shadowed with platinum, leaving tiny holes available for labeling. These holes are comprised of two portions (for diagrams, see Figs. 4, *C* and *D* and 8 in reference 29): (*a*) the side of the intramembrane

Resolution of label-fracture is high: 15 nm or less. Two results illustrate this resolution: (*a*) colloidal gold label is always 15 nm or closer to the edge of the fracture face; even in those cases, frequent presence of a cone of shadow associated with the gold particle and at a distance of 5–15 nm shows that this is genuine label associated to the very edge of the fracture face, where the outer half of the membrane has been cross-fractured (Fig. 1 *b*) (in freeze-fractured, unetched preparations such as ours, cross-fractured membranes of membrane halves are not visible); (*b*) colloidal gold labeling of particle aggregates (Fig. 7) is always within 10–15 nm of the line that demarcates the aggregate. Therefore, we conclude that colloidal gold label remains, after thawing and washing, at or very close to its original site. This can be explained only by permanence of the binding of the lectin-gold complex to the surface receptor at the outer surface of the membrane. Our results show that the label is not always associated to the particulate regions of the fracture face. For instance, in sperm cells a particle-free area of the plasma membrane at the base of the sperm head is heavily labeled. We presume that this label refers to glycolipids and/or peripheral membrane proteins (F. W. K. Kan and P. Pinto da Silva, manuscript in preparation).

In addition to providing an easy, reproducible method for labeling cell surfaces at high resolution, examination of label-fractured specimens will also allow the direct relation of the surface label to the freeze-fracture morphology of exoplasmic fracture faces, in particular to the distribution of intramembrane particles. When used in conjunction with complementary replicas, label-fractured specimens will also make it possible to directly relate the surface label to the distribution of intramembrane particles on protoplasmic fracture faces (F. W. K. Kan and P. Pinto da Silva, manuscript in preparation). These results may then be combined with those derived from the interpretation of fracture-labeled specimens, in particular the sidedness of membrane components and the existence and distribution of transmembrane proteins.

Clearly, label-fracture, introduced here, has a different scope and is not intended to substitute for the "fracture-label" technique that we have developed over the past few years. Each technique addresses different questions, although with a degree of overlapping (in both, a surface label can be related to a freeze-fracture image, albeit in different ways). Label-fracture is a technique to label cell surfaces: a paradox whereby routine surface labeling is approached via conventional freeze-fracture. The resolution and sensitivity of label-fracture can now be used to explore the ultrastructural patterns of distribution of cell surface receptors and their relation to membrane architecture.

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particle opposite to the source of platinum evaporation, and (*b*) contiguous with this, the smooth (bilayered) region of the membrane that is delimited by the cone of shadow. Therefore, access of the label to the fractured face becomes restricted to these small, bimodal spots. As most of the fractured face is now masked by a layer of platinum, any label at or in the proximity of these tiny openings (≤15 nm) is to be assigned to intramembrane particles. In the absence of interpretable results produced by labeled-replica methods, further discussion of its relative merits is premature.

P. Pinto da Silva dedicates this article to his parents, Drs. Antonio and Quitéria Pinto da Silva.

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