ADVANCES IN CELL BIOLOGY

A Research Annual

Editor: KENNETH R. MILLER

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INTRODUCTION

In the formal sense, cell biology is one of the youngest of biology's many subfields. International meetings on cell biology, formal organizations, and journals of cell biology are comparatively recent entries into the bewildering thicket of scientific disciplines. Yet cell biology is one of the oldest and most fundamental routes of inquiry into the nature of living systems. A justification for emphasizing the importance of cell biology is easy enough to develop. The cell is the basic unit of life, and detailed studies of cells, their structure, behavior, and their components are clearly required to develop a detailed appreciation of living organisms.

The techniques of research in cell biology are as varied as the imaginations of the investigators who still find the cell to be an intriguing target. From the original tool of cell biology, the light microscope, researchers have developed a toolbench that includes biological, biophysical, chemical, and molecular approaches to the understanding of cell structure and cell function. One of the reasons for the great vitality of cell biology at this period in the history of biology is precisely this diversity of approach and technique, and techniques developed for use in cell biology are now beginning to have wider applications in other fields of biology, medicine, and biotechnology.

In the series that is inaugurated with this volume we hope to capture some of the excitement and vitality that characterizes the state of the field today. We have chosen an approach in which individual researchers at the forefront of cell biology have been invited to highlight some of the most important research and technical advances in their research. While these articles will integrate these advances into the large picture of cell biology research, the authors have not been burdened with the task of reviewing all advances in their fields or assembling an exhaustive citation list from which the reader can develop a bibliography of all work published on a particular topic. Instead, we have tried to choose a format in which

individual researchers will do what they do best: to explain the areas in which they work and to highlight the research developments and advances which are shaping that field and influencing the direction of scientific thought and experimentation. In doing this, I hope that we will present to the scientific public a series that will improve communication and dialogue, and will bring to cell biology a focused annual series containing the scientific highlights of the past year.

Kenneth R. Miller
Series Editor

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THE ANALYSIS OF FRACTURED MEMBRANE "HALVES"

Knute A. Fisher

I. INTRODUCTION

The physical and chemical analysis of split membrane leaflets is based on several common principles and observations. First, it has been established that the process of freeze fracture cleaves the lipid bilayer of the membrane, separating the inner and outer lipid leaflets (Branton, 1966; Pinto da Silva and Branton, 1970). Second, the split leaflets or "halves," like intact membranes, are known to be composed of protein, lipid, and carbohydrate. Third, transmission electron microscopy (TEM) consistently reveals intramembranous particles (IMPs) in all biological membranes.[1] And finally, it is assumed that IMPs represent membrane-spanning polypeptides and/or lipid-polypeptide aggregates and that the surrounding smooth regions represent lipid monolayers.

Physical analyses of fractured membrane "halves" are usually based on electron microscopy. Freeze-fractured samples are shadowed with electron-

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scattering metals such as platinum-carbon, tungsten, or tantalum, and the surface replica is examined by TEM. Chemical information about split membranes is usually derived by correlation or by cytochemistry. Correlative studies include those in which treated and control cell membranes are examined for changes in IMP number or distribution. If the transmembrane or in-plane distribution of IMPs is found to be altered by the treatment, the morphological changes are often explained in molecular terms. More direct cytochemical approaches have also been developed, including methods to label lipids, carbohydrates, and polypeptides. For example, polyene antibiotics such as Filipin have been used to evaluate the cholesterol content of freeze-fractured membranes (Verkleij et al., 1973; Elias et al. 1979). Filipin combines with unesterified 3-β-hydroxysterols and forms large protuberances and pits in the fractured membranes. Labels for anionic lipids have also been developed (Bearer and Friend, 1980), and membrane carbohydrates are routinely investigated by lectin labels conjugated to microscopic markers such as ferritin or gold.

IMP labeling methods were first developed in the early 1970s to validate membrane splitting (Pinto da Silva and Branton, 1970), and they have been used to probe the surface distribution of lectin (Pinto da Silva and Nicolson, 1974) and antibody binding sites (Pinto da Silva et al., 1971). The distribution of the marker on the etched surface can be correlated with surface morphology and with the distribution of IMPs on contiguous fracture faces. In erythrocyte ghost membrane studies, pH-induced aggregation of IMPs aided the localization of ferritin conjugates relative to the particle clusters (Pinto da Silva, 1972).

Over the past several years the resolution of the labeling approaches has increased significantly. Now, in principle, individual IMPs can be labeled. In a recent method called "fracture-label" (Pinto da Silva et al., 1981), chemically and physically stabilized membranes are fractured, thawed, labeled with colloidal gold or ferritin conjugates, and processed for thin-section TEM. In the "sectioned labelled-replica technique" (Rash et al., 1982), replicated and labeled tissues are processed for thin-section TEM. In a more recent technique, dubbed "label-fracture" (Pinto da Silva and Kan, 1984), colloidal gold protein conjugates are used to label the outer surfaces (ES) of the membrane before fracturing. After freeze fracturing and shadowing, the position of the underlying gold relative to the IMPs can be evaluated. These methods are covered in some detail by Pinto da Silva (later chapter in this volume) and will not be discussed here further.

This brief review is focused on an approach to the analysis of fractured membranes that avoids chemical fixation and thus allows the examination of both native and probe molecules. This approach involves the preparation of highly oriented membrane monolayers by adsorption to planar surfaces. The planar membrane monolayers are freeze fractured, and the split mem-

brane fractions are analyzed either by electron microscopic autoradiography or by direct physical and chemical methods.

Several simple concepts and basic assumptions provide the foundation for planar membrane splitting. Electrostatic attachment of cells and membranes to a surface is based on the principle of attraction between the negatively charged cell surface and a positively charged support, e.g., polylysine-treated glass (Nevo et al., 1955; Fisher, 1975; Mazia et al., 1975) or Alcian Blue-treated mica (Sommer, 1977; Nermut, 1981). Cells have also been attached by aldehydes (Büechi and Bächi, 1979) or lectins (Alpin and Hughes, 1981), but such procedures will not be reviewed here. The formation of planar cell and membrane monolayers is based on the testable assumption that washing attached cells or shearing or sonicating attached membranes will remove unattached material. Central to planar membrane splitting is the assumption that the process of freeze fracture physically separates the two leaflets of the lipid bilayer (Branton, 1966). And, finally, the possibility for "half" membrane enrichment and isolation was derived from the observation that planar membranes lying parallel to the plane of fracture will fracture in preference to those oriented randomly (Fisher, 1975). This review will highlight recent advances in planar monolayer autoradiography and biochemistry and will provide a perspective for the contributions of these studies to the field of cell and membrane biology.

II. TECHNICAL ADVANCES

A. Polylysine-Treated Planar Surfaces

Because most cells and membranes are negatively charged at physiological pH, they will bind to surfaces carrying the opposite charge. Positively charged planar surfaces can be easily prepared by treating clean, negatively charged surfaces with cations. Mazia and co-workers (1975) prepared polylysine-treated, carbon-coated grids for electron microscopy studies. Clean glass, too, provides an excellent surface for adsorption of polylysine (Nevo et al., 1955; Fisher, 1975). Alcian Blue, a tetravalent cationic dye, has also been used to produce a positive charge on glass (Sommer, 1977) or mica (Nermut, 1981) surfaces for both microscopic and chemical studies. For the chemical studies undertaken in my laboratory, however, polylysine-treated glass has been used exclusively because the color and copper ions present in Alcian Blue interfere with some spectrophotometric and biochemical assays.

Planar preparations of cells and membranes are well suited to both spectroscopic and microscopic analyses (Fisher, 1981). In addition, they are useful for biochemical studies of molecular and transmembrane

topographies. The orientation and amount of membrane can be directly quantified by microscopy, and attached membranes can be chemically or enzymatically modified with precise control (for reviews see Fisher, 1978, 1980; Nermut, 1982, 1984).

B. Planar Cell Monolayers

A wide variety of cells have been attached to planar surfaces and examined by microscopy. Examples include *Halobacterium halobium* purple membranes (Fisher and Stoeckenius, 1977; Nermut and Williams, 1977), *Saccharomyces cerevisiae* (Maurer and Mühlethaler, 1981), *Dictyostelium discoideum* (Clarke et al., 1975), EL-4 mouse lymphoma cells (Bächi and Büechi, 1981), KB cells (Nermut, 1982), Vero cells (Nermut, 1982), macrophages (Aggeler and Werb, 1982), and red blood cells from humans (Fisher, 1975; Edwards et al. 1979) and sheep (Lang and Nermut, 1980).

Planar cell monolayers (Figure 1a) have been examined by a variety of light and electron microscopic techniques including scanning electron microscopy, thin section TEM, surface shadowing, negative staining, and freeze fracture (Figure 1b). Cell monolayers are especially useful for the enrichment and isolation of the plasma membrane and, in membrane splitting studies, for isolating the outer leaflet of the membrane bilayer. Single membrane monolayers, on the other hand, are especially useful for the enrichment and isolation of inner leaflets.

C. Planar Membrane Monolayers

Planar membrane monolayers are flattened single membranes attached to a planar surface. They are thus composed of both leaflets of the lipid bilayer. Membrane monolayers can be formed using isolated membrane sheets, such as purple membrane, or intact cells, such as human erythrocytes (Figure 2a), given that they have been washed well to remove soluble proteins, which compete for surface binding sites. The formation of bona fide single membrane monolayers (SMM) requires methods that are sample dependent; each new preparation should be verified by light and electron microscopy. For example, single sheets of *H. halobium* purple membrane can be attached, as a function of pH, to polylysine-treated glass (PLG) by either their extracellular (Figure 2b) or cytoplasmic (Figure 2c) surfaces. To remove aggregated, overlapped, or folded membranes, the preparations must be briefly sonicated (Fisher, 1981). In contrast, SMM of attached intact cells, ghosts, or vesicles are usually formed by hydraulic shearing. This process has been used to expose the cytoplasmic surface of the membrane for microscopic examination of cytoskeletal structures (Clarke et al., 1975). Nermut (1982) dubbed his version of the method "lysis-squirting," and I have referred to it as "hydraulic shearing" (Fisher, 1982a).

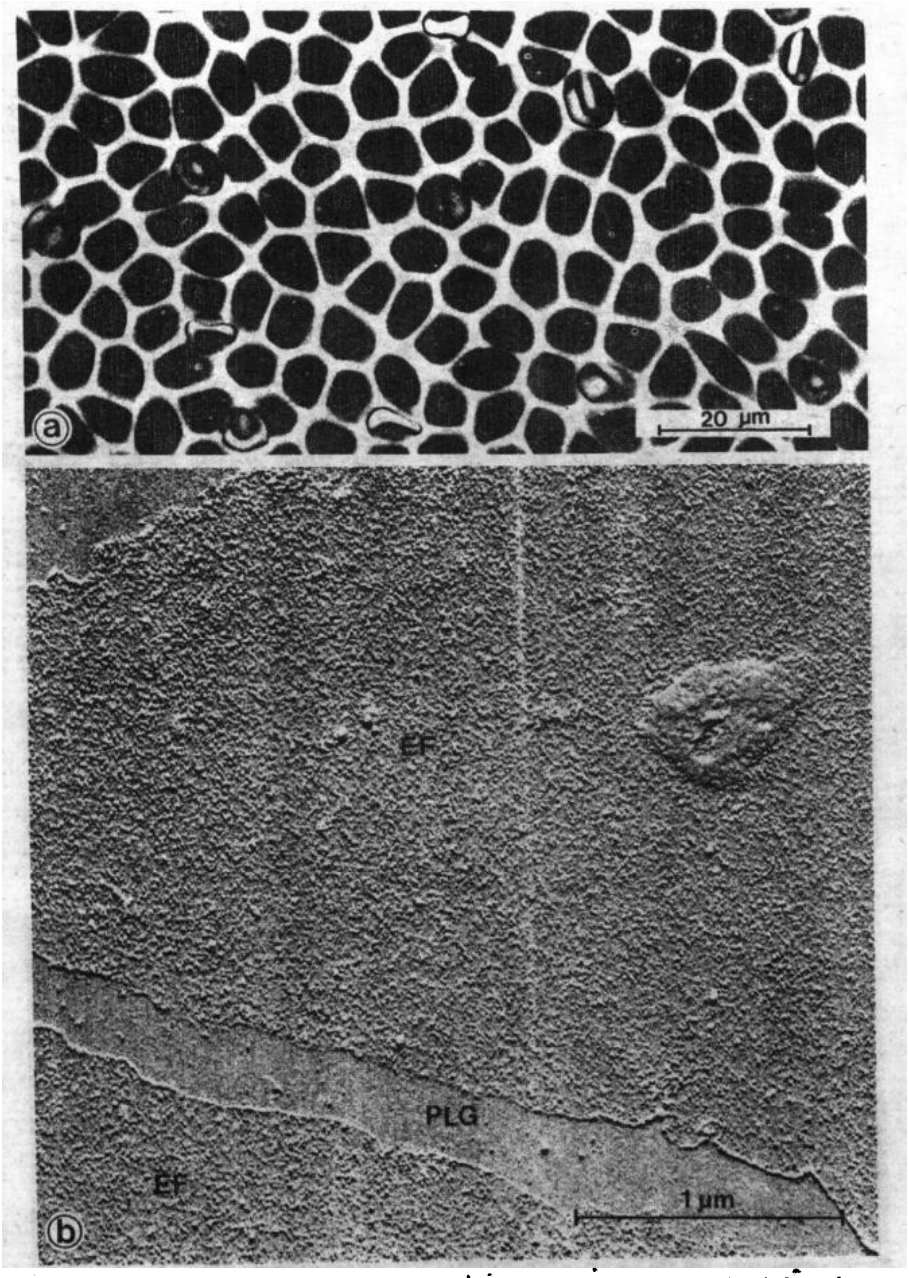

Figure 1. Planar cell monolayers of human erythrocytes. (a) Light micrograph of a monolayer of intact erythrocytes attached to a polylysine-treated glass surface. (b) Electron micrograph of a freeze-fractured erythrocyte monolayer. Split extracellular membrane "halves," E-faces (EF), on polylysine-treated glass (PLG). Platinum carbon shadowing direction for all micrographs, from bottom to top.

Figure 2. Electron micrographs of single membrane monolayers. (a) Hydraulically sheared human erythrocyte membranes, fixed with glutaraldehyde-osmium, dehydrated with ethanol-acetone, dried with nitrogen gas,

Biochemical studies, especially, require confirmation that hydraulically sheared surfaces retain single planar membranes that are neither overlapped nor folded. Verification can be provided both by light microscopy and by TEM of shadowed freeze-dried planar surfaces. For example, if cell monolayers are "sheared," the removal of cellular contents plus all unattached membrane should expose only the inner cytoplasmic surfaces (PS) of single membranes. Shearing allows chemical or enzymatic modification of an exposed stabilized cytoplasmic surface and provides a means to enrich for the cytoplasmic leaflet of the membrane in membrane splitting studies (discussed below in Section I.E).

D. Monolayer Freeze Fracture of Cells

Cells cultured in Petri dishes often grow to confluence, forming "cell monolayers." Such monolayers are useful for freeze-fracture studies because the fracture plane often passes through the cell as well as along the attached planar plasma membrane (Collins et al., 1975); but they are poorly suited to biochemical studies, which require square centimeter areas of flattened cell membranes. Experimental efforts to flatten membranes by applying them to planar surfaces arose from the observation that membrane bilayers oriented parallel to the plane of fracture split in preference to those of more random orientation (Figure 1b) (Fisher, 1975). Flattened cell membrane monolayers are prepared by applying cells to cleaned planar glass surfaces treated with polylysine. The monolayer is sandwiched against a second glass or copper sheet, then frozen, and fractured. The split preferentially passes through the attached cell membranes.

In biochemical studies planar cell monolayer splitting is used to evaluate the transmembrane distribution of native or probe molecules. By forming flattened sheets it is possible to enrich for the outer leaflet of the plasma membrane. Whereas initial studies required microscopic methods to quantify splitting, improved surface-labeling methods were soon devised (Figure 3). Labeling the extracellular surface of the red cell with fluorescent lectins made it possible to quantify rapidly the amount of outer surface and thus the fraction of outer leaflet of split membrane (Fisher, 1982a). Similarly the

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Figure 2—continued

and shadowed with platinum-carbon. (b) Purple membrane fragments attached by their extracellular surfaces. Pitted cytoplasmic surfaces (PS) are exposed. (c) Purple membrane fragments attached by their cytoplasmic surfaces. Cracked extracellular surfaces (ES) are exposed. In this preparation 8% of all membranes are oriented with the PS exposed.

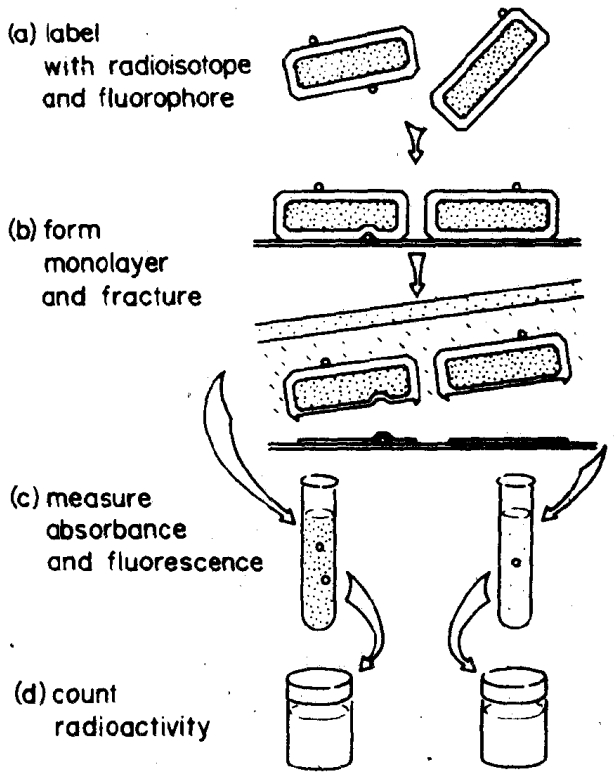

Figure 3. The method of double-labeled membrane splitting (DBLAMS). (a) Washed radio-labeled erythrocytes are surface-labeled with FITC-concanavalin A. (b) Cells are applied to polylysine-treated coverglasses forming a planar cell monolayer that is sandwiched against another piece of glass, frozen, and fractured. (c) The two split fractions are dissolved in buffered detergent solutions, and hemoglobin absorbance and fluorescein fluorescence are measured. (d) Samples are transferred to vials for measurement of radioactivity by LSC. (Reproduced from Fisher, 1982a.)

inner leaflet fraction could also be rapidly quantified by hemoglobin absorbance; the Soret band provides a sensitive spectrophotometric marker of the fraction of cytoplasmic surface. Although the idea of cell monolayer freeze fracture is simple, its application to quantitative biochemical analyses requires careful attention to manipulative detail, avoidance of contamination, and correction for unexpected phenomena such as the enhancement of fluorescence by trace amounts of hemoglobin (Fisher, 1982a).

The quantity of split membrane lipids and proteins produced by fracturing planar surfaces is small. For example, only 5×10^6 red blood cells (RBCs)

can bind to an 11 × 22 mm polylysine-treated coverglass. Given 5.7×10^{-7} mg membrane protein per red cell ghost (Steck, 1974), there is a total of 2.8 μg membrane protein per 11 × 22 mm glass. Hydraulic shearing further reduces the amount of bound membrane to 50% or less, reducing total membrane protein to 1.4 μg or less. Individual membrane-associated proteins such as actin are thus reduced to nanogram levels, which require highly sensitive methods of detection. Fortunately more sensitive methods continue to be developed, and there are now many techniques to measure nanogram quantities of both lipids and polypeptides.

Cell monolayer freeze fracture has been used to examine the bilayer distribution of both lipids and proteins. For example, in early experiments the transbilayer distribution of native cholesterol was examined using membrane-splitting methods and was verified and quantified by light and electron microscopy (Fisher, 1976a). Native cholesterol in split membrane fractions was measured at the nanogram level by the combination of thin layer chromatography, charring, and microdensitometry. The transbilayer distribution of cholesterol in the intact cell was found to be asymmetric: there was more cholesterol in the outer leaflet than in the inner leaflet of the bilayer (Table 1). The cholesterol study represented the first application of membrane splitting to the biochemical analysis of a molecule of biological interest. The quantitative microscopic approach, although direct, is time consuming, and quantitation requires knowledge or measurement of the total surface area of the cell. Both of these limitations were countered by development of the "doubled-labeled membrane splitting" (DBLAMS) method (Figure 3).

DBLAMS provides a rapid, quantitative biochemical method for analyzing split membrane fractions of whole cell monolayers. It requires having quantifiable markers for each side of the bilayer. This was accomplished for the human erythrocyte by using the fluoresceinated lectin FITC-concanavalin A as a label for the extracellular surface and native hemoglobin as a marker for the inner leaflet (Fisher, 1982a). The quantities of extracellular and cytoplasmic leaflets are thus measured by fluorescence and absorbance spectroscopy. Once the portion of each leaflet in each of the split membrane fractions has been determined, the transbilayer distribution of native and probe molecules can be calculated. For example, in a test study, the transmembrane distribution of radioiodinated concanavalin A was found to be asymmetric. Essentially all of the label was detected on the extracellular surface (ES) of the membrane and partitioned with the extracellular face (EF) upon splitting. In recent studies we have used DBLAMS to determine the transbilayer distribution of tritiated cholesterol. Our initial results suggest that the tritiated probe mimics the distribution of the native molecules and is also asymmetrically distributed. More label is found associated with the extracellular leaflet than with the cytoplasmic leaflet of the bilayer.

Table 1. An Example of the Application of Cell Monolayer Membrane Splitting to an Analysis of the Transbilayer Distribution of Native Cholesterol in Human Erythrocyte Membranes

The ratio of cholesterol in the extracellular leaflet of the bilayer relative to that in the cytoplasmic leaflet (outside/inside) is consistently greater than 1. Thus, cholesterol is asymmetrically distributed across the red cell bilayer, more in the outer leaflet than in the inner leaflet.

		Cholesterol (ng)			*Ratio*[d]
Exp.[a]	*Transparent (%)*[b] (f_t)	*Cu side* (M_B)	*Glass side* (M_t)	*Total* (M_A)	*Outside/Inside* (m_o/m_i)
PBS 1	88 ± 3 (4)	358 ± 24 (7)	130 ± 25 (7)	488	1.02
2	88 ± 5 (4)	333 ± 60 (3)	143 ± 21 (3)	476	1.64
3	91 ± 8 (4)	350 (1)	125 (1)	475	1.36
TBS 4	87 ± 6 (3)	246 ± 43 (7)	133 ± 13 (7)	379	3.34
5	85 ± 7 (3)	240 ± 37 (8)	128 ± 27 (7)	368	2.39
6	91 (1)	210 ± 32 (4)	86 ± 11 (4)	296	2.01
7	90 (1)	206 ± 8 (4)	96 ± 5 (4)	302	2.75

[a] Exps. 1–3, erythrocytes in 310 imosM phosphate-buffered saline (PBS); Exps. 4–7, in 340 imosM Tris-buffered saline (TBS).

[b] Area determined by planimetry after lipid extraction, drying, and photographic enlargement; mean ± SD. Numbers in parentheses; cover-glasses extracted and planimetered.

[c] Data not vertically comparable: final solvent volume varied among different experiments. Numbers in parentheses; thin-layer chromatography spots examined. Total indicates the arithmetic sum of copper plus glass. In Exps. 6 and 7, control data of bound but unfractured [293 ± 54 (3) and 288 ± 28(3), respectively] and of unbound and unfractured [335 ± 37 (4) and 313 ± 23 (4)] were obtained.

[d] Ratio calculated by applying the analysis given in Fig. 7 using tabular data from f_t, M_A, M_B or M_t, plus $f_{ts} = 0.33$ (determined in earlier experiments); m_i and m_o of cholesterol computed for each experiment and their ratio, m_o/m_i, given here. Mean ratio for PBS experiments = 1.34 ± 0.31; for TBS experiments = 2.62 ± 0.57; and for both (overall) = 2.07 ± 0.81.

Source: Reproduced from Fisher (1976a).

In principle, the transbilayer distribution of polypeptides can also be evaluated by DBLAMS. In practice, however, sample preparation procedures for methods such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) have interfered with spectrophotometric and fluorometric assays (Fisher and Yanagimoto, 1986). Nevertheless, DBLAMS has shown that freeze fracture of intact erythrocyte membranes does not produce new polypeptide bands; i.e., no new bands can be detected after SDS PAGE by staining with Coomassie Blue or with silver, or by fluorography of lactoperoxidase radioiodinated polypeptides (Figure 4) (Fisher and Yanagimoto, 1986). In other words, freeze fracture does not cleave mem-

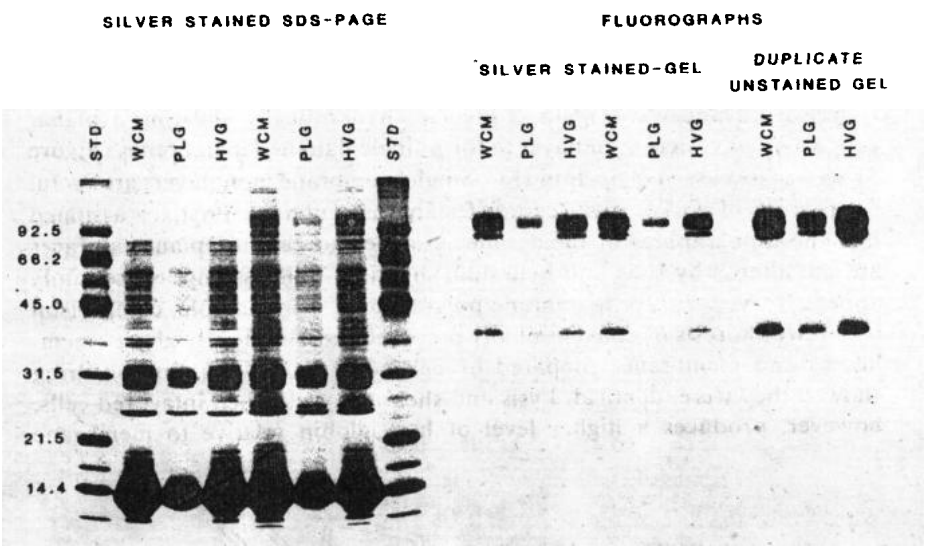

Figure 4. Gel and fluorographs of lactoperoxidase radioiodinated intact and freeze-fractured human red cells. Silver-stained gel (left), its matching fluorograph (center), and a fluorograph of a duplicate unstained gel (right). Gel lanes show duplicate sets of unsplit whole cell monolayers (WCM), split complementary fractions enriched in extracellular (PLG) and cytoplasmic (HVG) membrane "halves" and molecular weight standards (STD) indicated in kilodaltons. Radioactive regions of the left gel were excised for LSC with the center fluorograph used as a template. Note that all bands in the split fractions (PLG and HVG) have matching counterparts in the unsplit control (WCM) whether unlabeled or labeled. Radiolabeled bands include, from top to bottom: band 3 (the anion channel); glycophorin A dimer (PAS 1, just below band 3) and monomer (PAS 2). (Reproduced from Fisher and Yanagimoto, 1986.)

brane-spanning proteins of the human red cell. Moreover, covalent bonds remain intact regardless of the degree of glycosylation. Neither highly glycosylated molecules like glycophorin A, more than 60% of whose mass is composed of carbohydrate (Furthmayr, 1981), nor less glycosylated molecules like the anion channel band 3 are fragmented. Importantly, in planar cell monolayer freeze-fracture studies intrinsic cell-membrane interactions are preserved until splitting. These studies also have a significant weakness, however: only a small percentage of the polypeptides in the split fractions is derived from split membrane. There is a high background of unsplit membrane. As discussed below, this problem was circumvented by the development of SMM splitting methods.