

## Use of the Translational Mobility of a Plasma Membrane Protein to Assess Fertilization of Mouse Oocytes and Viability of Mouse Zygotes and Two-Cell Embryos<sup>1</sup>

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### ABSTRACT

The fluorescence photobleaching recovery (FPR) technique was used to measure the translational mobility of a glycoprotein recognized by the monoclonal antibody (mAb) S75 in plasma membranes of mouse oocytes, zygotes, and two-cell embryos. Glycoprotein fractional mobility (*f*) was significantly decreased in membranes of unfertilized oocytes compared to zygotes or two-cell embryos (*f* values,  $46 \pm 2$  and  $65 \pm 2\%$ , respectively;  $p < 0.0001$ ). Reduced apparent glycoprotein mobility was also observed in morphologically degenerated zygotes and two-cell embryos compared to viable zygotes and two-cell embryos (*f* values,  $8 \pm 1$  and  $60 \pm 3\%$ , respectively;  $p < 0.0001$ ). These results indicate that the FPR technique can be used to assess oocyte fertilization and preimplantation embryonic viability. This method may be useful in the evaluation of embryonic viability following in vitro fertilization and in the detection of toxic effects of novel compounds on embryonic development.

### INTRODUCTION

Frye and Edidin [1] first demonstrated that some proteins are capable of diffusing over micrometer-scale distances in the plasma membrane. This observation led to considerable interest in measuring the diffusivities of membrane proteins, because of the possible involvement of translational diffusion in a wide variety of cellular functions [2–7]. Introduction of the technique of fluorescence photobleaching recovery (FPR) made possible the direct quantitation of translational diffusion [8–11]. This technique involves measuring the fluorescence of a membrane probe excited by a laser beam focused to a small spot on the cell surface. A fraction of probe molecules in the illuminated region is then photochemically destroyed by a brief, intense laser pulse. If the fluorescently labeled membrane components are free to translate in the plane of the membrane, the level of fluorescence in the illuminated region is observed to recover toward the initial value [6]. This type of experiment allows calculation of both the fraction of probe molecules that are free to diffuse in the plasma membrane and the diffusion coefficient of the mobile probes [8].

Fertilization is rapidly followed by changes in the composition, physical properties, and function of the egg's plasma

membrane [12, 13]. A number of investigators have examined the effect of fertilization on the translational and rotational diffusion of molecules in the plasma membrane in an attempt to correlate changes in the functional status of the egg with changes in the physical state of the plasma membrane [14–19]. By electron spin resonance, Campisi and Scandella [14] found a slight decrease in the order parameter of sea urchin oocyte membrane lipids after fertilization. By FPR, various lipid probes manifested either an increase, a decrease, or no change in translational diffusion after fertilization of eggs of the sea urchin *Strongylocentrotus purpuratus* [17]. Johnson and Edidin [16] observed dramatic slowing of both protein and lipid diffusion in mouse egg plasma membranes upon fertilization. Wolf et al. [17] did not find a general effect of fertilization on the diffusion of mouse egg membrane glycoproteins labeled with succinyl concanavalin A (S-ConA), but they did observe an increased diffusion coefficient (*D*) and a decreased fractional mobility (*f*) for other proteins labeled with trinitrobenzene sulfonate (TNBS) [19].

Assessment of oocyte fertilization and embryonic viability remains a problem for reproductive biologists. There are no direct indicators of cellular viability. Investigators of cell death have therefore turned to indirect indicators that suggest whether cells are healthy or nonviable [20]. Many methods of evaluation have been used [21–23], including morphological assessment [24], biochemical and cell biological methods [25–31], and genetic approaches and biophysical methods [32–36]. Cell proliferation [37] and <sup>51</sup>Cr uptake assays [38, 39] have also been used to assess viability.

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Cytoskeletal rearrangements are likely to underlie at least some of the morphological changes associated with cell death. Since changes in the translational diffusion of plasma membrane glycoproteins have been associated with cytoskeletal alterations [40, 41], it was hypothesized that the translational mobility of labeled cell surface proteins could be used as an accurate measure of the viability of preimplantation embryos. In the present study the FPR technique was used to quantify the translational diffusion of a specific glycoprotein, recognized by the monoclonal antibody (mAb) S75, in the plasma membranes of mouse oocytes, zygotes, and two-cell embryos.

## MATERIALS AND METHODS

### *Mouse Ovulation and Fertilization*

Virgin females (CF1 strain), 35 to 42 days old, were used to provide oocytes, zygotes, and two-cell embryos. Intra-peritoneal injection of gonadotropin in the form of eCG (5 IU/0.1 ml; Sigma, St. Louis, MO), followed 48 h later by hCG (from human pregnancy urine, 5 IU/ml; Sigma), was used to induce ovulation. For experiments requiring fertilized zygotes and two-cell embryos, BGSJLF/J male proven breeders were used for mating immediately after injection of CF1 female mice with hCG.

### *Removal of Oocytes, Zygotes, and Two-Cell Embryos*

Female mice were killed by cervical dislocation. Oocytes or one-cell embryos (zygotes) were flushed from the ampullary region of the oviduct on the day after injection of hCG or injection of hCG followed by mating, respectively. Adherent cumulus cells were removed with 0.1% hyaluronidase (Sigma) digestion. Cells containing two pronuclei and/or two or three polar bodies were judged to be fertilized. Two-cell embryos were flushed from the oviduct with Whitten's medium 20 h postcoitum. Fifteen to twenty-five oocytes, zygotes, or two-cell embryos were obtained from each mouse and used in FPR and two-dimensional fluorescence imaging studies.

Morphologically normal and degenerated zygotes and two-cell embryos were cultured to the blastocyst stage in microdroplets of Whitten's medium. Degenerated zygotes and embryos were defined by the presence of rough, vacuolated cytoplasm, of a fragmented nucleus, and of a polygonal, asymmetric plasma membrane. All morphologically degenerated zygotes and two-cell embryos remained in the one- and two-cell stages, respectively, whereas 80–90% of zygotes and two-cell embryos with normal morphology entered the blastocyst stage.

### *S75 (HSA-10) mAb*

The WHO Workshop monoclonal IgG antibody S75 [42, 43] was shown to react with a glycoprotein (unpublished data; WHO Sperm Antigen Workshop, 1986, Toronto,

ON, Canada) on the surface of preimplantation mouse embryos and was used for translational mobility and fluorescence imaging studies. S75 was obtained by standard methods after immunization of mice with live acrosome-reacted human sperm [42] and was selected on the basis of its reactivity to methanol-fixed and unfixed human sperm [43]. S75 was also shown to cross-react with human placenta. Detailed study of the S75 mAb and its cognate glycoprotein antigen (designated STX-10) from human sperm and placenta is in progress.

### *Fluorescein-5-Isothiocyanate Labeling of S75 mAb*

Briefly, a 20-fold molar excess of fluorescein-5-isothiocyanate (FITC; Sigma) was dissolved in 400  $\mu$ l dimethylformamide (DMF; Sigma). A 1/9 volume of DMF was added to 10 ml of a solution of purified S75 mAb in PBS. The FITC/DMF solution was then added drop-wise to the mAb solution, with continued stirring, over 15 min at 0°C. This solution was stirred at room temperature for 2.5 h. Unreacted FITC was removed by gel filtration using a PD-10 column (Sephadex G10; Pharmacia, Piscataway, NJ). Protein was eluted using PBS at pH 8 as previously described [44].

### *FITC-S75 Labeling of Oocytes, Zygotes, and Two-Cell Embryos for FPR and Two-Dimensional Fluorescence Imaging Studies*

Oocytes, zygotes, and two-cell embryos were washed and resuspended in Whitten's medium and mixed with one microdroplet of FITC-S75 solution (100:1, vol:vol) for 3 min. Cells were then washed five times in Whitten's medium. A microdroplet containing 20  $\mu$ l of Whitten's medium and 3–5 oocytes, zygotes, or two-cell embryos was transferred to a microscope slide. A coverslip was lowered onto the cells; cold cream (Nivea) was used around the edge of the coverslip as a spacer to prevent crushing of the sample.

In parallel experiments, a group of 50 morphologically viable two-cell embryos was exposed to FITC-S75 solution (100:1, vol:vol) for 3 min, washed five times in Whitten's medium, and cultured to the blastocyst stage. The proportion of FITC-S75-treated two-cell embryos that developed successfully to the blastocyst stage (40 of 50 embryos) was not significantly different ( $p = 0.26$ , contingency table analysis) from that for an untreated control group of two-cell embryos (45 of 50 embryos).

### *Labeling of Cells with Fluorescent Lipid Analogues for FPR and Two-Dimensional Fluorescence Imaging Studies*

Cells were labeled with several different lipid probes, including fluorescein phosphatidylethanolamine (Fl-PE), 1,2-acyl-2-(*N*-4-nitrobenzo-2-oxo-1,3-diazole)-phosphatidylethanolamine (NBD-PE), NBD-phosphatidylcholine (NBD-PC) (Avanti Polar Lipids, Alabaster, AL), and a series of 3,3'-diacylindocarbocyanine iodide (Di-I) probes including C14Di-I, C16Di-I, C18Di-I, and C22Di-I (Molecular Probes, Eugene,

TABLE 1. Translational mobility of FITC-S75-labeled glycoproteins in plasma membranes of mouse oocytes, zygotes, and two-cell embryos.\*

Sample	D	f	n
Oocytes	3.4 ± 0.4 <sup>a</sup>	46 ± 2 <sup>b</sup>	29
Zygotes, two-cell embryos <sup>†</sup>	2.6 ± 0.5 <sup>a</sup>	65 ± 2 <sup>b</sup>	26

\*Samples were labeled as described in *Materials and Methods* and translational mobility was measured by FPR: D, diffusion coefficient, × 10<sup>9</sup>·cm<sup>2</sup>·s<sup>-1</sup>; f, fractional mobility, %; n, number of independent measurements. Values are expressed as mean ± SEM. Statistical significance of differences between D and f values was determined by Student's two-tailed t-test: <sup>a</sup>not significant; <sup>b</sup>p < 0.0001.

<sup>†</sup>Results from zygotes and two-cell embryos were pooled because these two samples manifested identical diffusion coefficients and fractional mobilities.

OR). Probes were dissolved in PBS at various concentrations, as follows: Fl-PE, 0.2 to 20 000 ng/ml; NBD-PE, 0.2 to 20 000 ng/ml; NBD-PC, 0.2 to 20 000 ng/ml; C14Di-I, 0.2 to 20 ng/ml; C16Di-I, 0.2 to 20 ng/ml; C18Di-I, 0.2 to 20 ng/ml; C22Di-I, 0.2 to 20 ng/ml. Oocytes, zygotes, and two-cell embryos were labeled with the lipid probes at room temperature for various incubation times ranging from 1 min to 30 min. After labeling, cells were washed three times with Whitten's medium containing 1% BSA (Sigma). Labeled cells were mounted on slides as described above.

#### FPR

FPR was used to measure the translational mobility of labeled proteins and lipids [17, 18, 45, 46]. Briefly, a Gaussian laser beam was focused to a waist at the sample plane of a fluorescence microscope. After a brief, intense photobleaching pulse, recovery of fluorescence was monitored by periodic low-intensity pulses. Recovery resulted from the translational diffusion of unbleached fluorophores into the bleached area. Nonlinear least-squares analysis of fluorescence recovery data yielded both the diffusion coefficient (D; calculated from the rate of fluorescence recovery) and the fraction (f) of fluorescently labeled molecules that were free to diffuse into the previously bleached area on the time scale of the experiment [8, 47].

All data were recorded at room temperature. Bleaching pulses were typically 100 msec in duration using a beam power of 110 μW. Measuring pulses were 4 μsec in duration using a typical beam power of 10 μW. Measuring intensities and amplification settings were adjusted such that the signal from unlabeled cells was 1% of the signal from labeled cells. Fluorescence recovery was typically monitored for 50 sec. The Gaussian beam radius was 1.30 ± 0.16 μm as determined by scanning 0.22-μm-diameter fluorescent beads (Polysciences Inc., Warrington, PA) immobilized in saturated Airvol U-205 (Air Products, Allentown, PA) in glycerol and calculating the average 1/e<sup>2</sup> radius of the fluorescence profile of 20 beads. All data were taken and analyzed on a Meridian Instruments (Okemos, MI) ACAS 570 interactive laser cytometer. Each fluorescently labeled oo-

TABLE 2. Translational mobility of FITC-S75-labeled glycoproteins in morphologically viable and nonviable zygotes.\*

Sample	D	f	n
Viable zygotes	2.5 ± 0.5	60 ± 3 <sup>a</sup>	32
Nonviable zygotes	— <sup>†</sup>	8 ± 1 <sup>a</sup>	12

\*See legend to Table 1 for sample preparation and explanation of symbols.

<sup>†</sup>D cannot be determined for f < 20%.

<sup>a</sup>Statistical significance of differences between f values was determined by the Mann-Whitney U-test. p < 0.001.

cyte, zygote, or two-cell embryo was used for one FPR measurement.

#### Two-Dimensional Fluorescence Imaging

Two-dimensional fluorescence imaging of oocytes, zygotes, and two-cell embryos was also performed with the ACAS 570 interactive laser cytometer. Cells were scanned as the computer-controlled microscope stage moved through a prescribed x-y pattern over the fixed excitation laser beam. Fluorescence measurements were taken at 0.4-μm intervals using a typical beam power of 10 μW.

#### Statistical Analysis

Statistical analysis was performed on a Macintosh IICI computer (Apple Computer Inc., Cupertino, CA) using StatView II statistical software (Abacus Concepts, Inc., Berkeley, CA). Data were analyzed by the two-tailed unpaired Student's t-test in the case of balanced design (Table 1) and by the Mann-Whitney U test in the cases of unbalanced design (Tables 2 and 3).

## RESULTS

#### Labeling of Oocytes, Zygotes, and Two-Cell Embryos with Fluorescent Lipid Probes

Significant autofluorescence was not observed in oocytes, zygotes, or two-cell mouse embryos. Despite the normal appearance of oocytes, zygotes, and embryos by phase-contrast microscopy, all fluorescent lipid probes tested—including Fl-PE, NBD-PE, NBD-PC, C14Di-I, C16Di-I, C18Di-I, and C22Di-I—appeared to be translationally immobile (f < 10%) in both unfertilized eggs and fertilized zygotes and

TABLE 3. Translational mobility of FITC-S75-labeled glycoproteins in cells of morphologically viable and nonviable two-cell embryos.\*

Sample	D	f	n
Viable embryos	1.4 ± 0.1	40 ± 6 <sup>a</sup>	23
Nonviable embryos	— <sup>†</sup>	8 ± 1 <sup>a</sup>	8

\*See legend to Table 1 for sample preparation and explanation of symbols.

<sup>†</sup>D cannot be determined for f < 20%.

<sup>a</sup>Statistical significance of differences between f values was determined by the Mann-Whitney U-test. p < 0.001.

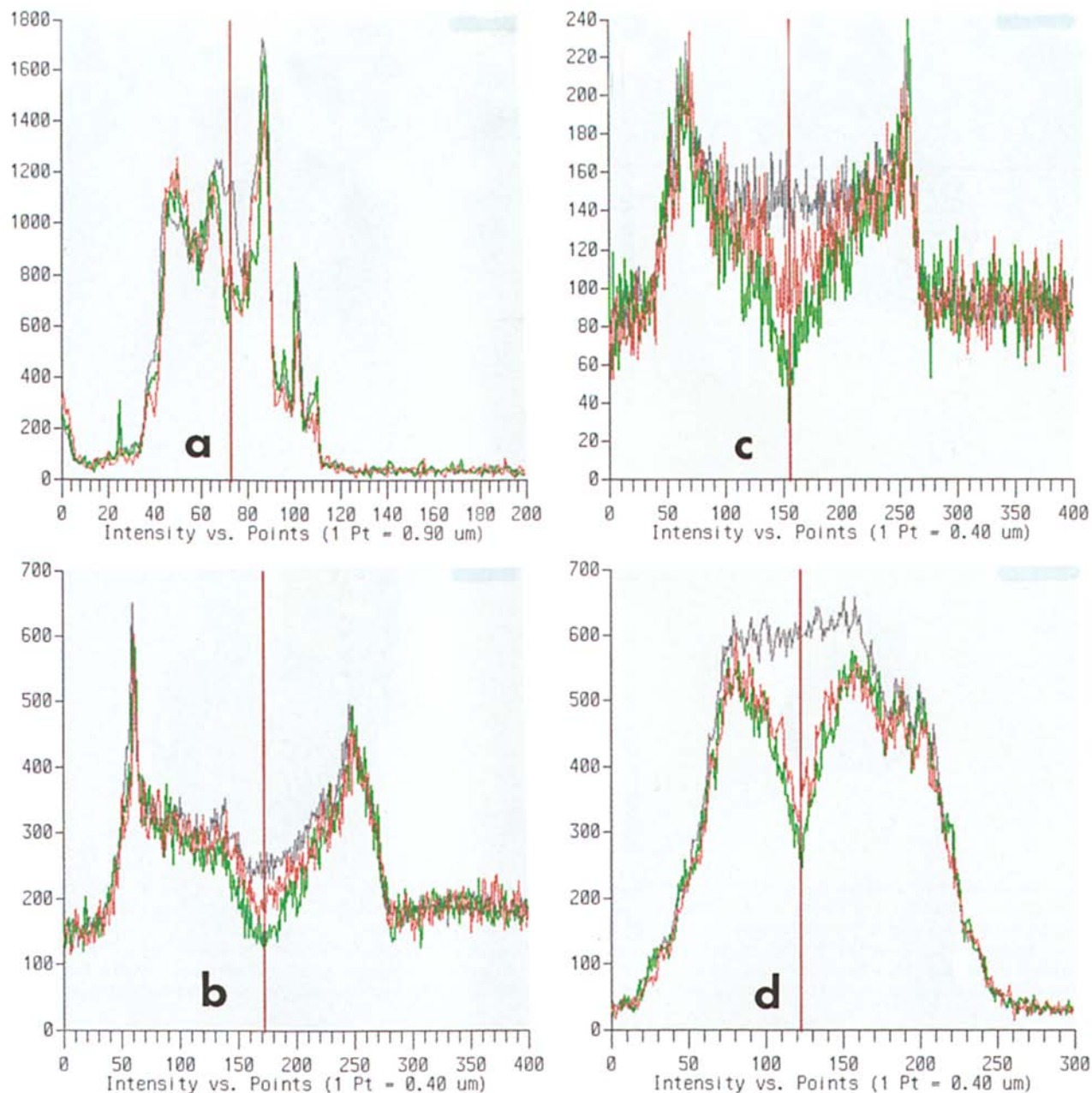


FIG. 1. FPR experiments. Individual fluorescently labeled mouse oocytes, zygotes, and two-cell embryos were scanned at 1.2-sec intervals in one dimension along a line passing through the diameter of the cell(s). Abscissa, position along the scan axis ( $\mu\text{m}$ ). Ordinate, fluorescence intensity (arbitrary units). Black scan: point-by-point average of the three scans taken prior to the bleaching pulse. Green scan: scan taken immediately following the bleaching pulse. Vertical line marks position of the bleached spot. Red scan: final scan taken at time = 50 sec after the bleaching pulse. Fluorescence recovery is indicated by return of the final (red) scan profile from the post-bleach (green) profile toward the pre-bleach (black) profile. Fractional mobility ( $f$ ) and diffusion coefficient ( $D$ ) were calculated from a nonlinear least-squares fit of the data taken during successive scans at the bleach position. Diffusion coefficients cannot be obtained accurately for  $f < 20\%$ . a) Viable FI-PE-labeled zygote:  $f$ ,  $< 10\%$ ;  $D$ , not measurable. FI-PE appeared to be translationally immobile, probably due to probe internalization (see Fig. 2a). b) Viable FITC-S75-labeled zygote:  $f$ ,  $37\%$ ;  $D$ ,  $2.7 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ . c) Viable FITC-S75-labeled two-cell embryo:  $f$ ,  $61\%$ ;  $D$ ,  $2.5 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ . S75-labeled proteins manifested significant translational mobility in the plasma membranes of viable zygotes and two-cell embryos (see Fig. 2b). d) Nonviable FITC-S75-labeled zygote:  $f$ ,  $< 20\%$ ;  $D$ , not measurable. FITC-S75 appeared to be translationally immobile in nonviable zygotes and two-cell embryos, probably due to probe internalization (see Fig. 2, c and d).

two-cell embryos (Fig. 1a). In all cases, two-dimensional fluorescence images showed the lipid probe fluorescence to be localized in the oocyte, zygote, or embryo cytoplasm rather than at the plasma membrane (Fig. 2a).

*Effect of Fertilization on Mobility and Distribution of FITC-S75*

FPR was used to measure the translational mobility of FITC-labeled S75 in oocytes, zygotes, and two-cell embryos.

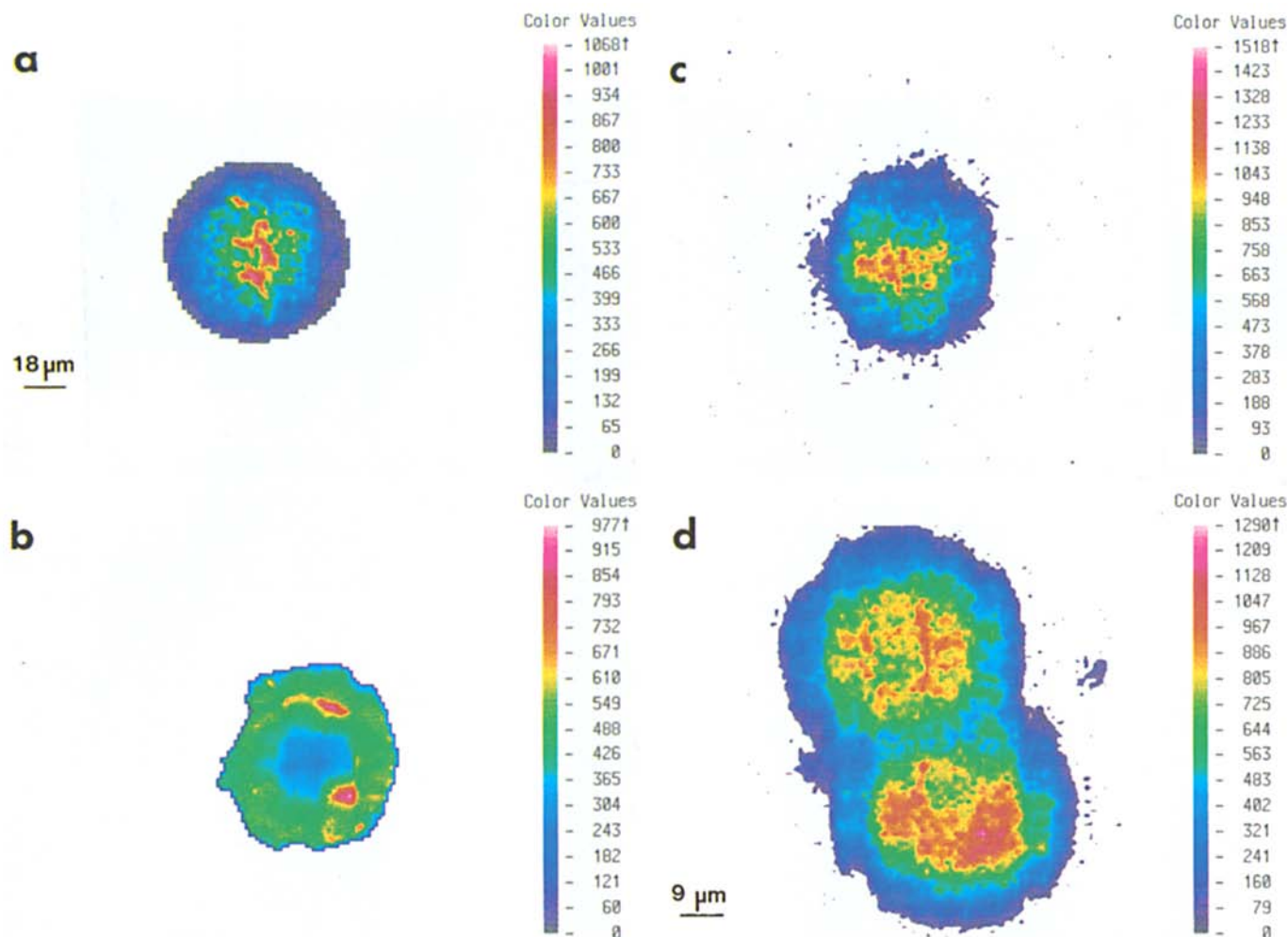


FIG. 2. Two-dimensional fluorescence imaging experiments. Individual fluorescently labeled mouse oocytes, zygotes, and two-cell embryos were scanned in two dimensions as described in *Materials and Methods*. Color values (arbitrary units) represent intensity of labeling with the fluorescent probe, from little or no labeling (black/violet) to marked labeling (red/pink). Distance scale for a, b, and c is  $0.6 \text{ cm} = 18 \mu\text{m}$ ; scale for d is  $0.6 \text{ cm} = 9 \mu\text{m}$ . a) Viable FI-PE-labeled zygote. FI-PE was rapidly internalized by oocytes, zygotes, and two-cell embryos. b) Viable FITC-S75-labeled zygote. The FITC-S75 probe labeled proteins in the plasma membranes of viable oocytes, zygotes, and two-cell embryos. The probe was not internalized by viable cells. c) Nonviable FITC-S75-labeled zygote. d) Nonviable FITC-S75-labeled two-cell embryo. FITC-S75 was rapidly internalized by nonviable zygotes and two-cell embryos.

The phase-contrast image of each cell was examined carefully for the presence or absence of the second pronucleus and for the distinct presence of two polar bodies. FITC-S75 manifested significantly greater  $f$  in zygotes and two-cell embryos than in unfertilized oocytes (Table 1). In contrast, the  $D$  of FITC-S75 was not significantly different between the two samples (Table 1). By two-dimensional fluorescence imaging, FITC-S75 was localized on the cell surface both before and after fertilization.

#### *Effects of Embryonic Viability on Mobility and Distribution of FITC-S75*

Significant differences were observed in the fractional mobilities of FITC-S75 in morphologically viable vs. nonviable zygotes (Table 2; Fig. 1, b and d) and two-cell em-

bryos (Table 3; Fig. 1c). In both cases, morphologically nonviable cells manifested apparently immobile FITC-S75. One-dimensional fluorescence scans and two-dimensional fluorescence images were also significantly different for viable vs. nonviable zygotes and two-cell embryos (Figs. 1, b-d and 2, b-d and 2, b-d). Morphologically viable oocytes, zygotes, and two-cell embryos displayed fluorescence profiles in which the most intense fluorescence was detected over the plasma membrane (Figs. 1, b and c, and 2b). In contrast, morphologically damaged or degenerated zygotes (Figs. 1d and 2c) and two-cell embryos (Fig. 2d) displayed fluorescence images in which the most intense fluorescence was recorded over the cytoplasm of the degenerated cells. It therefore appeared that FITC-S75 was restricted to the plasma membrane of the viable cells but entered the cytoplasm of the nonviable cells.

## DISCUSSION

### *Fertilization Studies*

The FPR technique was used to measure the translational mobility of a glycoprotein recognized by the mAb S75 in plasma membranes of mouse oocytes, zygotes, and two-cell embryos. A significant increase in glycoprotein f was observed in zygotes and two-cell embryos compared to unfertilized oocytes. These data suggest that dramatic changes occur in the plasma membrane during fertilization of the oocyte and early development of the embryo.

Fertilization results in a complex series of metabolic changes. The most prominent fertilization-associated changes in sea urchin eggs include modifications in cellular structure, increases in respiration rate and coenzyme content, increases in substrate uptake rates, and transient proteolytic activity. These changes may be markers for the activation of enzymes that synthesize the lipids, proteins, and nucleic acids required for cell division and differentiation [48]. Other changes that occur during fertilization include increased intracellular pH, sodium influx, liberation of calcium ions from intracellular stores, modification of existing membrane proteins, and incorporation of new lipids and proteins into the plasma membrane [12]. Given this constellation of fertilization-associated changes, it might be expected that the physical properties of the oocyte plasma membrane would also change at fertilization, reflecting the transformation of the cell from an inactive to an active state.

Others have shown that the effect of fertilization on the D of plasma membrane lipids depends on lipid probe structure [18] and acyl chain length [17]. For some probes D was increased, for some probes D was decreased, and for some probes D did not change in sea urchin [18] and mouse [17] eggs upon fertilization. In the present system, all of the lipid probes used (including Fl-PE, NBD-PC, NBD-PE, C14Di-I, C16Di-I, C18Di-I, and C22Di-I) were rapidly internalized, possibly as a result of the high metabolic activity of mouse oocytes, zygotes, and two-cell embryos. It was therefore not possible to examine experimentally the effect of fertilization on plasma membrane lipid mobility in mouse oocytes. The differences between the lipid probe internalization rates obtained in the present study and those reported in earlier studies [17, 18] could be due to different experimental conditions. The observation here that all lipid probes were rapidly internalized contrasts with the finding that S75-labeled proteins remained on the cell surface of viable oocytes, zygotes, and two-cell embryos. These data may indicate that S75-labeled proteins are excluded from domains in the plasma membrane that are rapidly turning over.

The effect of fertilization on the translational mobility of plasma membrane proteins is likely to depend on the particular species of protein examined. In other systems, Johnson and Edidin [16] found, upon fertilization, a decrease in the D of proteins labeled with Fab fragments of rabbit anti-

mouse embryo Ig. Wolf and Ziomek [19] observed an increase in the D and a decrease in the f of TNBS-labeled proteins, and no change in the mobility of S-ConA-labeled proteins, upon fertilization. Here, an increase in the f of S75-labeled proteins was found upon fertilization of mouse oocytes, without change in D. Several interpretations are consistent with these varying data. Fertilization could affect interactions of some but not all membrane glycoproteins. Increased f could result from fertilization-induced dissociation of "lateral" interactions (e.g., aggregation) among specific transmembrane glycoproteins or of "vertical" interactions between transmembrane proteins and cytoskeletal proteins or extracellular matrix components. Alternatively, synthesis of new translationally mobile transmembrane proteins or shedding of immobile proteins upon fertilization could yield an increase in protein f. Finally, disruption of micrometer-scale lipid domains could cause a selective increase in the f of proteins present in such domains. These mechanisms could vary in importance among different populations of transmembrane proteins and different cell types, resulting in diverse effects of fertilization on membrane protein and lipid mobility in different biological systems.

### *Viability Studies*

Assessments of embryonic development and viability are currently based on widely accepted morphological criteria [24]. The FPR and two-dimensional fluorescence imaging studies reported here on viable and degenerated mouse zygotes and two-cell embryos were designed to test the hypothesis that the molecular mechanisms restricting transmembrane protein mobility are altered upon cell death. In FPR studies, a significant decrease in the f of S75-labeled proteins was observed upon embryonic degeneration. Two-dimensional imaging studies indicated, however, that this decrease was not due to increased association between transmembrane glycoproteins and protein or lipid components of the plasma membrane. Rather, it appeared either that S75-labeled proteins were rapidly internalized in morphologically degenerated zygotes and two-cell embryos or that the plasma membranes of such cells were not competent to prevent S75 mAb entry into the cytoplasm. In either case, the present studies indicate that changes in the translational mobility of S75-labeled glycoproteins provide a sensitive indicator of embryonic viability. The quantitative fluorescence techniques reported here could be useful in determining the fertilization status of mouse oocytes for research purposes and in detecting toxic effects of novel compounds or other experimental conditions on embryonic development. Further, the demonstration that the FITC-S75 probe does not adversely affect development of two-cell embryos to the blastocyst stage suggests that quantitative fluorescence-based assays employing similar probes can be considered for use in assessing the viability of embryos following *in vitro* fertilization.

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