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ULTRASTRUCTURAL ANALYSIS OF DYNAMIC CELLULAR PROCESSES: A SURVEY OF CURRENT PROBLEMS, PITFALLS AND PERSPECTIVES

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Abstract

Dynamic phenomena in cells that can be analyzed on the ultrastructural level comprise so different aspects as ion shifts, conformational changes of macromolecules, membrane particle rearrangements, lipid phase transitions, protein-protein interactions (notably ligand-receptor interactions, including their sorting and sequestration), reversible membrane-to-membrane contacts, membrane fusions, transcellular transport phenomena, restructuring of cytoskeletal elements, ciliary and flagellar beat, cell shape changes, etc. Only some of these phenomena can be analyzed under stationary conditions, while others are unidirectional and sometimes very rapid. Therefore, the methodical approaches to be used (primary methods and follow-up procedures) might be widely different. Quite different methods are available, such as fast freezing, specific labeling, low temperature processing and/or analysis, x-ray-microanalysis, etc. Only occasionally are there alternative non-ultrastructural control methods available. This survey paper tries to analyze the degree of reliability (or uncertainty) of current methods and to pinpoint the goals and eventually also new methodical perspectives for an integrative approach to analyze dynamic cellular processes with the high temporal and spatial resolution provided by the electron microscope.

KEY WORDS: Cells, cryofixation, cryomethods, dynamics, electron microscopy, freeze-fracturing, freeze-substitution, membranes, organelles, ultrastructure

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Introduction

Dynamic cellular processes can be subgrouped according to the speed of the events involved (though this is not always precisely known). With some of the fastest events (e.g., conformational changes of macromolecules), however, one can also consider extreme states without resolving individual processes. The "time resolution" (c.f. Plattner and Bachmann 1982, Knoll et al. 1987) required to "catch" dynamic events is accordingly quite different. (The same holds true for "spatial resolution".) Resolution has to be considered not only for analytical but also for preparative procedures. Further progress in the analysis of cell dynamics will greatly depend on new methodical developments. "Fast methods" frequently also proved important to circumvent preparative artifacts entailed by "slow methods" (chemical fixation, e.g.), such as organelle distortion and vesiculation.

Stationary phenomena will be considered separately. For instance, electron microscopic (EM) techniques, such as image filtering and object reconstruction, have greatly contributed to an understanding of the dynamics of intercellular communication via gap-junctions (Unwin and Ennis 1984). Many of the immuno- and affinity-labelings also belong to this group of techniques, though the phenomena involved are of moderate speed. They, thus, can also be analyzed under pulse-labeling conditions. Again recent EM-work has greatly contributed to our understanding of receptor-ligand interactions and intracellular trafficking of the organelles involved (Willingham et al. 1981; Geuze et al. 1984). New preparation methods, such as ultracryotomy (Tokuyasu 1983), low temperature embedding (Carlemalm et al. 1985) or labeling with gold particles of defined size classes (Slot and Geuze 1985) etc., have allowed for this progress.

As a prototype of very fast dynamic processes one can consider membrane fusions. "Fast methods" appear mandatory to get insight into such processes (Knoll et al. 1987). Since they must be expected to occur on the millisecond time scale or less (Siegel 1984, 1986), cryofixation is the only possible EM-method. For

a long time one faced the problem, e.g., to analyze membrane fusion during exocytosis. One either has to synchronize these events (Plattner 1987a) or to perform analyses on a statistical level (Schmidt et al. 1983). Current cryofixation techniques (allowing for rapid freezing of native biological materials) are surveyed by Plattner and Bachmann (1982), Robards and Sleytr (1985), Gilkey and Staehelin (1986), Menco (1986) and Sitte et al. (1987). Most provide sufficiently high cooling rates to analyze fast processes; however, mere ultrastructural preservation is not a sufficient criterion (Knoll et al. 1987).

Fast processes, occurring in the time range of a second (fractions or multiples thereof), are represented, for instance, by muscle contraction, ion shifts, ciliary beat, endocytosis, organelle shape changes, etc. There has accumulated an increasing body of evidence for the occurrence of considerable artifacts, when such phenomena are analyzed by "standard" chemical fixation (Plattner 1981, Knoll et al. 1987), so that again cryofixation, followed by freeze-fracturing or freeze-substitution, appears advisable.

Slow processes are, e.g., restructuring of cytoskeletal elements or some of the organelle and cell shape changes. Although this is the least problematic group of dynamic phenomena, artifacts - avoidable though - may occur even on this level (Cheng and Reese 1985; Sandoz et al. 1985a).

For each individual problem one has to find, eventually to develop, the most appropriate method. Fast freezing has become highly important not only with regard to time resolution, but also as a means to make just "this one step behind the scene" which chemical fixation does not allow for. Since this has been postulated (Plattner et al. 1973), many artifacts have been published and many of them can be ascribed to the non-critical adherence to "conventional" (mostly chemical, hence slow) preparation methods. The book by Rash and Hudson (1979) contains a collection of examples. Although this aspect is now generally accepted, other new methods designed to analyze dynamic cellular events now have to be purged from similar unexpected pitfalls. Just two most urgent examples: Though affinity- and immunolabelings became indispensible tools, the kind of affinity marker (Farquhar 1978, Davies et al. 1981, Gonatas et al. 1984) or of immuno-label (Helenius and Mellman 1983, Ukkonen et al. 1983) affects results with regard to kinetics and targetings in the course of intracellular transport. Another valuable new method is microinjection. Since it has only rarely been combined with EM-analysis (Wehland and Willingham 1983), we are hardly aware of potential pitfalls, which are now gradually recognized (Momayezi et al. 1986 and unpublished observations, McClung et al. 1987)

Only in some instances can one resort to alternative methods, like cell fractionation, vital staining, fluorescence energy transfer, nuclear magnetic resonance, x-ray-diffraction, etc. Some of them face similar problems as EM studies. Examples are the redistribution of soluble proteins (during cell fractionation (Scheele et al. 1978) or microinjection (see above)) which now should be avoidable by the latest developments of EM techniques. This will bring us to a solution of challenging novel problems. In this sense EM techniques develop to an increasingly powerful integrative approach for the analysis of cell dynamics with high temporal and spatial resolution.

Analysis of dynamic processes

Cryofixation: State of a most important art

The analysis of fast processes requires, of course, "fast methods". Cryofixation is the only approach principally possible (at least until other techniques such as, e.g., microwave "fixation" (Chew et al. 1983, Login et al 1986), have proven to represent a reasonable alternative). It is available in widely different variations (for reviews, see Plattner and Bachmann 1982, Robards and Sleytr 1985, Gilkey and Staehelin 1986, Menco 1986, Sitte et al. 1987) and each of them might be particularly suitable for a particular problem. It can yield cooling rates of 10^4 - $>10^5$ K/s (Plattner and Bachmann 1982, Bachmann and Mayer 1987), and, thus, very high time resolution values. Values actually required for analyzing fast processes are discussed in more detail by Knoll et al. (1987). Since some dynamic phenomena, like lipid phase transitions or membrane fusion might occur at temperatures well above 273 K, high cooling rates right at the beginning of the cooling process are required (Plattner and Knoll 1984, Knoll et al. 1987). This might be provided by rather simple methods like a 1-side propane jet (Plattner and Knoll 1984).

High pressure freezing (Moor 1987) available for small tissue pieces does not appear appropriate for investigating fast processes (Sitte et al. 1987), but experimental proof for this or the opposite assumption is not yet available.

Only since a few years we know that the highest cooling rates available allow for a true vitrification of water from the liquid phase (Mayer and Brüggeller 1982, Dubochet et al. 1982, Bachmann and Mayer 1987, Dubochet et al. 1987); but conditions to be used (either vigorous injection of a fine jet or dipping of an extremely thin film) are either so harsh, that maximal freezing velocities cannot be expected ever to be achieved with fragile biological materials (Bachmann and Mayer 1987), or the size of the biological material is limited to objects of subcellular size (Stewart and Vigers 1986, Dubochet et al. 1987). It is also important that devitrification occurs already at 140 K (Bachmann and Mayer 1987). Since most currently available follow-up procedures (freeze-etching, -substitution and -drying) require higher temperatures (Robards and Sleytr 1985, Steinbrecht and Zierold 1987), recrystallization artifacts have to be expected, at least on a small scale (Bachmann and Mayer 1987). As discussed below, this must have implications for current discussions on the hypothesis on the microtrabecular

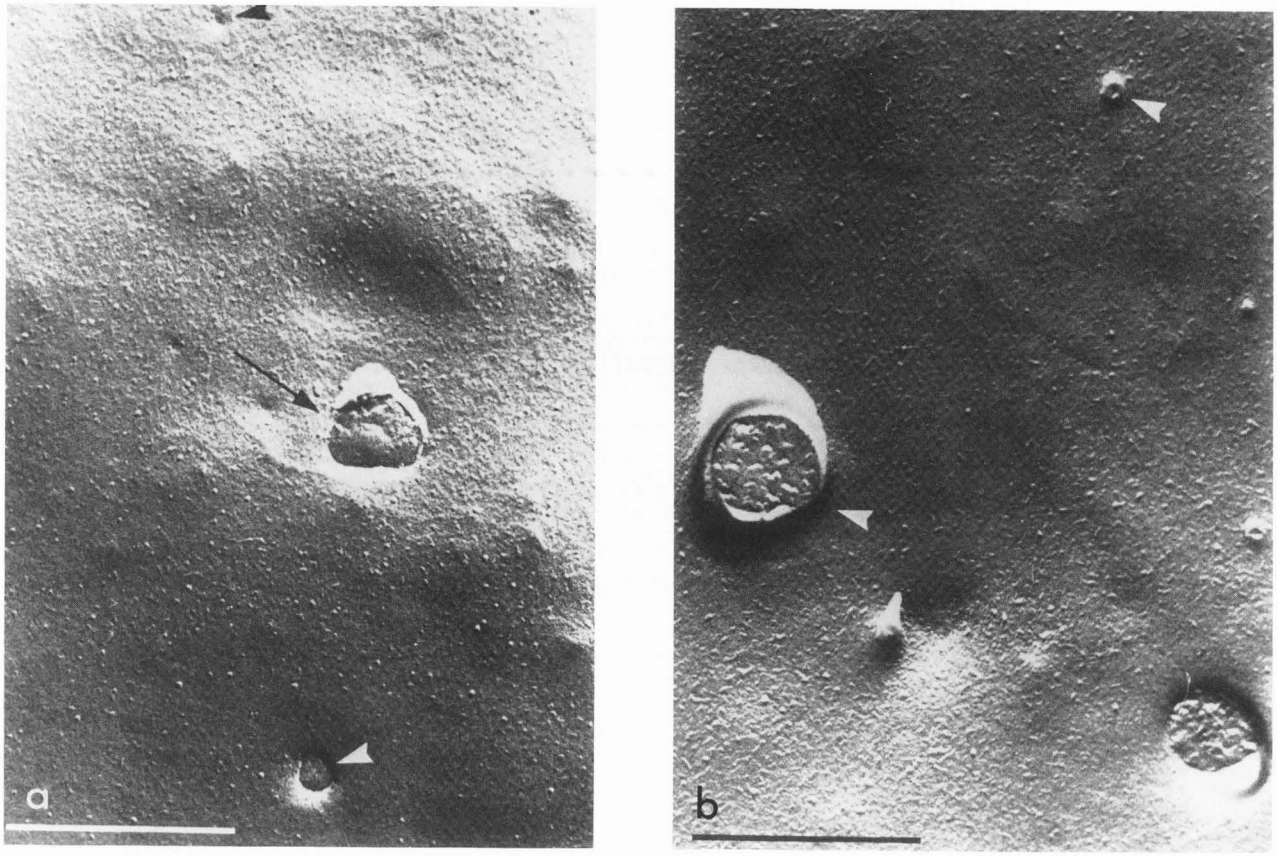

Fig. 1: Freeze-fractured plasma membranes of chromaffin cells rapidly frozen during stimulation with carbachol. Exo-endocytotic openings of a variety of size classes are visible, marked by arrows and arrowheads. While the larger openings show continuity of the aqueous compartments, the smallest structures visible might represent local perturbations of the membrane bilayer.
a) Protoplasmic fracture face, b) exoplasmic fracture face. Bar = 0.5 µm. (From Schmidt et al. 1983, with permission of the publisher)

organization of the cytosol (Wolosewick and Porter 1979, Porter and Anderson 1982).

Very fast processes

Membrane fusions. Since estimates of the time required for the actual process of membrane fusion are in the range of 0.1 - 1 milliseconds (Siegel 1984, 1986), it was not surprising that the earliest fusion stages could not be detected for a long time on freeze-fracture replicas (Tanaka et al. 1980). Just the best cooling rates obtained (Plattner and Bachmann 1982, Knoll et al. 1987) might be sufficient and it is also important to provide such high cooling rates already right at the beginning of the cooling process (Plattner and Knoll 1984). Using such methods (propane-jet sandwich freezing) Schmidt et al. (1983) were able to "catch" the smallest early fusion stages (10 nm in size) ever observed, in a biological membrane, during exocytosis in chromaffin cells (see Fig. 1). These stages might correspond to inverted micelles of the kind proposed by Verkleij (1984) and DeKruijff et al. (1985) as fusion intermediates, but local stochastic deviations from the lipid bilayer structure would also appear possible (Bearer et al. 1982, Boni et al. 1984). Though these data corroborate the "focal fusion concept" (Plattner 1981, 1987b) with important biological implications (see below), currently available ultrastructural (or any other) methods do not allow us to resolve the precise arrangement of lipids in the actual fusion zone, of course.

There are highly temperature sensitive lipids, the structure of which can be ascertained only by fast freezing from above phase transition temperatures (Ververgaert et al. 1973, Van Venetie and Verkleij 1981) as confirmed by low temperature x-ray-diffraction and differential scanning calorimetry (Gulik-Krzywicki and Costello 1978, Melchior et al. 1982). Thermotropic phase separations as related "freezing artifacts" may occur due to insufficient cooling rates in membranes containing mixtures

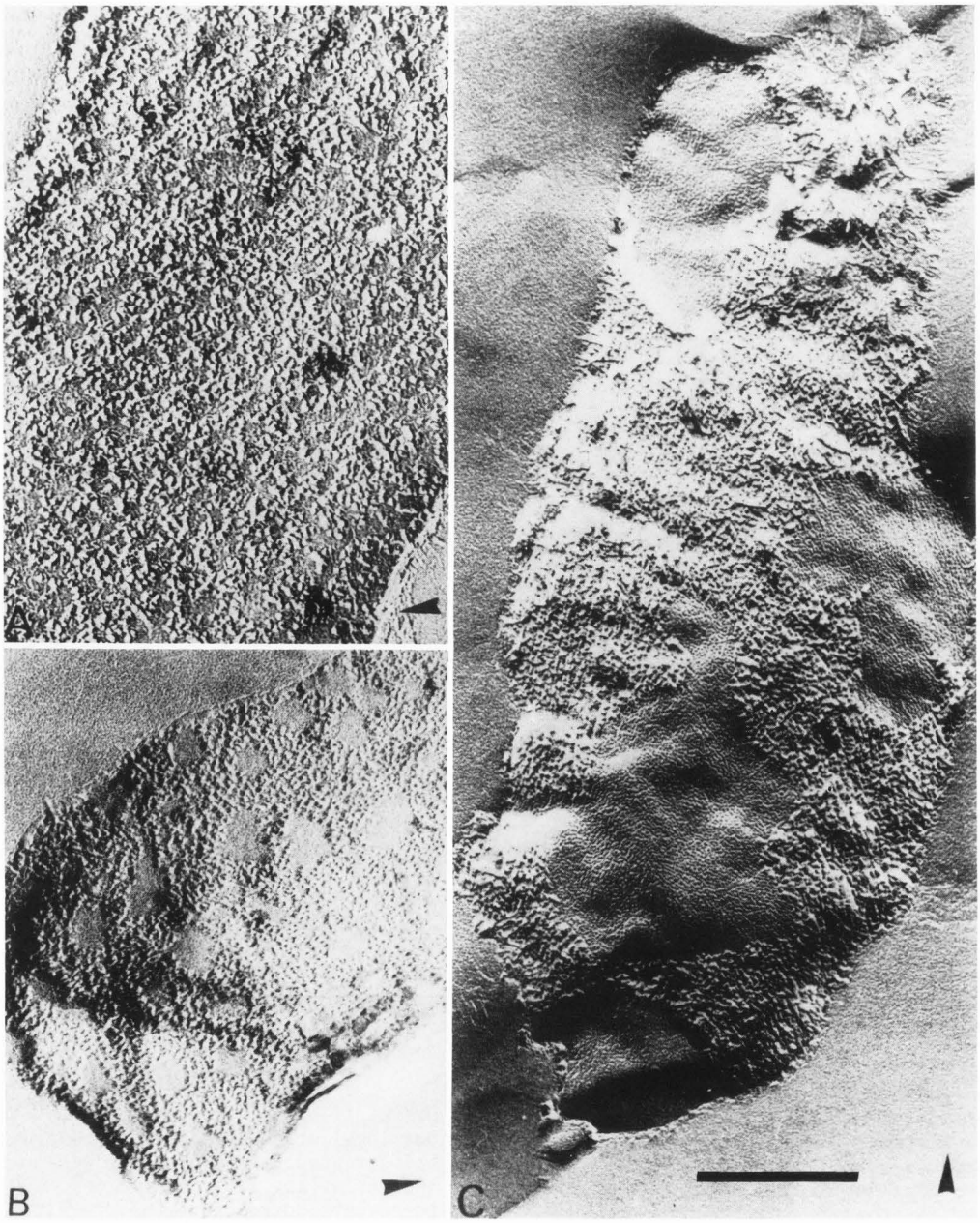

Fig. 2: Freeze fractured plasma membranes of Escherichia coli K1060 rapidly frozen from above (a), within (b) and below the lipid phase transition temperature (as revealed by differential scanning calorimetry). Segregation of lipid and protein upon solidification of the membrane lipids (as visible in (b) and (c)) may occur also due to insufficient cooling rates during freezing from above the transition temperature.
Bar = 0.2 µm. (From: Verkleij and Ververgaert 1975, with permission of the authors and of the publisher)

of lipids with different phase transition temperatures (Verkleij and Ververgaert 1975). In biological membranes this may be the cause that membrane intercalated particles are "squeezed out" from smooth lipid patches (Wunderlich et al. 1973, Kleemann and McConnell 1974, Verkleij and Ververgaert 1975) as illustrated in Fig. 2. In this case the requirement of very high cooling rates is demanded by the high lateral mobility of the membrane lipids, which is orders of magnitudes higher than that of membrane proteins. Lateral diffusion of membrane proteins has been captured even with rather moderate cooling rates after experimentally induced clustering (Schuler et al. 1978, Sowers and Hackenbrock 1981). Clustering of membrane particles can also be achieved by a pH shock (PintoDaSilva 1972) or by adding calcium (Verkleij and Ververgaert 1975, Schober et al. 1977); this might have some bearing on the subsequent subject.

Exocytosis. The occurrence of similar phenomena in freeze-fractured stimulated secretory cells has later been shown to be due to the use of "conventional" chemical preparation techniques (before freezing), (Orci et al. 1981, Plattner 1981), possibly combined with a lipid fluidization effect occurring under conditions of exocytosis triggering (Chandler 1984). With regard to exocytosis regulation, fast freezing methods (followed by freeze-fracturing and -substitution) have yielded important new insights (nerve terminals: Heuser et al. 1979, Heuser and Reese 1981; oocytes: Chandler and Heuser 1979; mast cells: Chandler and Heuser 1980; amoebocytes: Ornberg and Reese 1981; chromaffin cells: Schmidt et al. 1983; thrombocytes: Morgenstern et al. 1987). In all cases, fusion was seen to start with a rather small opening (also see above) without the formation of a diaphragm (Plattner 1981, 1987b). This is opposite to the scheme derived from chemical fixation (Palade 1975). Furthermore, freeze-fracture replicas revealed no shift of integral or peripheral membrane proteins before fusion occurs (Plattner 1981, 1987b); this was again opposite to results obtained with previously used "standard techniques" (Orci et al. 1981). It was important to find out the "true" sequence of ultrastructural events during exocytosis, since only then the question arose, what role proteins might play during this process; subsequently this important aspect could be systematically analyzed (Plattner et al. 1987).

Recent calculations (Sitte et al. 1987) have shown that - using cold metal surface techniques - laborious and expensive work with liquid helium is not mandatory, though this has occasionally been inferred from work on transmitter release (Heuser et al. 1979). It now appears more important to pinpoint quite precisely the actual plane within a nerve terminal, if one wants to judge correctly the mode and time course of exo-endocytotic processes, since these are delayed in deeper layers of the object (Torri-Tarelli et al. 1985). Another artifact hazard inherent to slam freezing methods has been discussed by PintoDaSilva and Kachar (1980) who pointed out that delicate structures of possible relevance for exocytosis might be damaged by the strong impact.

Fast freezing methods, especially when combined with freeze-substitution, would appear particularly suitable to solve the notorious question (Finkelstein et al. 1986) of vesicle swelling during exocytosis. EM evidence for its occurrence is available so far only from recent work with thrombocytes (Morgenstern et al. 1987, see Fig. 3), but the authors - correctly - also envisage microfusions (difficult to detect) followed by swelling of vesicle contents as an alternative explanation. Clearly more work, perhaps with the application of exogenous markers (Van Putten et al. 1987) to discriminate between swelling before and after (micro-?) fusions, would be desirable.

Fast processes

Muscle contraction. While the sliding filament hypothesis had been derived in 1953-1963 from static EM pictures (Huxley 1963), a decade later it was possible by fast freezing and freeze-substitution to visualize the extreme functional states of contraction and relaxation (Van Harreveld et al. 1974). One more decade was needed to establish accompanying ion shifts, again by cryofixation, but then followed by ultracryotomy, freeze-drying and x-ray microanalysis (Wendt-Gallitelli and Wolburg 1984).

Ion shifts. The last mentioned example shows that, although ion shifts occur rapidly, they can be tackled by recent methodical developments. X-ray microanalysis and electron energy loss analysis are applicable to native materials subjected to rapid freezing, cryosectioning and freeze-drying (Somlyo 1985, Somlyo et al. 1985). Even liver tissue surfaces could be appropriately frozen (by forced contact with melting Freon) and analyzed for ion distributions. This revealed the endoplasmic reticulum, rather than mitochondria, as physiological calcium stores (Somlyo et al. 1985). Unfortunately, transient changes of free intracellular calcium as a second messenger are beyond the reach of such methods (Somlyo 1985). Recently Schmitz and Zierold (personal commun.) were able to analyze ion shifts in secretory contents of paramecia during rapid synchronous exocytosis (50 millisecond events over 1 s; Plattner 1987a) which was "caught" by fast freezing.

Ciliary and flagellar beat operate many times per second. Therefore cryofixation is an appropriate method for "fixing" metachronal beat waves (Wooley 1974, Barlow and Sleigh 1979); this can avoid inconsistencies which otherwise often are obtained (Barlow and Sleigh 1979) when using even optimally adapted chemical fixation methods (Parducz 1967). It would now be interesting to push analyses further into more subtle details, such as - what we would like to call - the "torsion beat hypothesis" by Omoto and Kung (1980) or the question of conformational changes of dynein arms (Heuser 1981; however, see below).

Mitochondria as seen in live cells are "extremely dynamic organelles capable of profound changes in size, form, and location" (Izagaloff 1982). Shape and volume changes in situ are linked to respiration and phosphorylation, as shown with time-lapse cinematography (Frederic 1958). Structural transitions between "orthodox" and "condensed" stages, dependent on

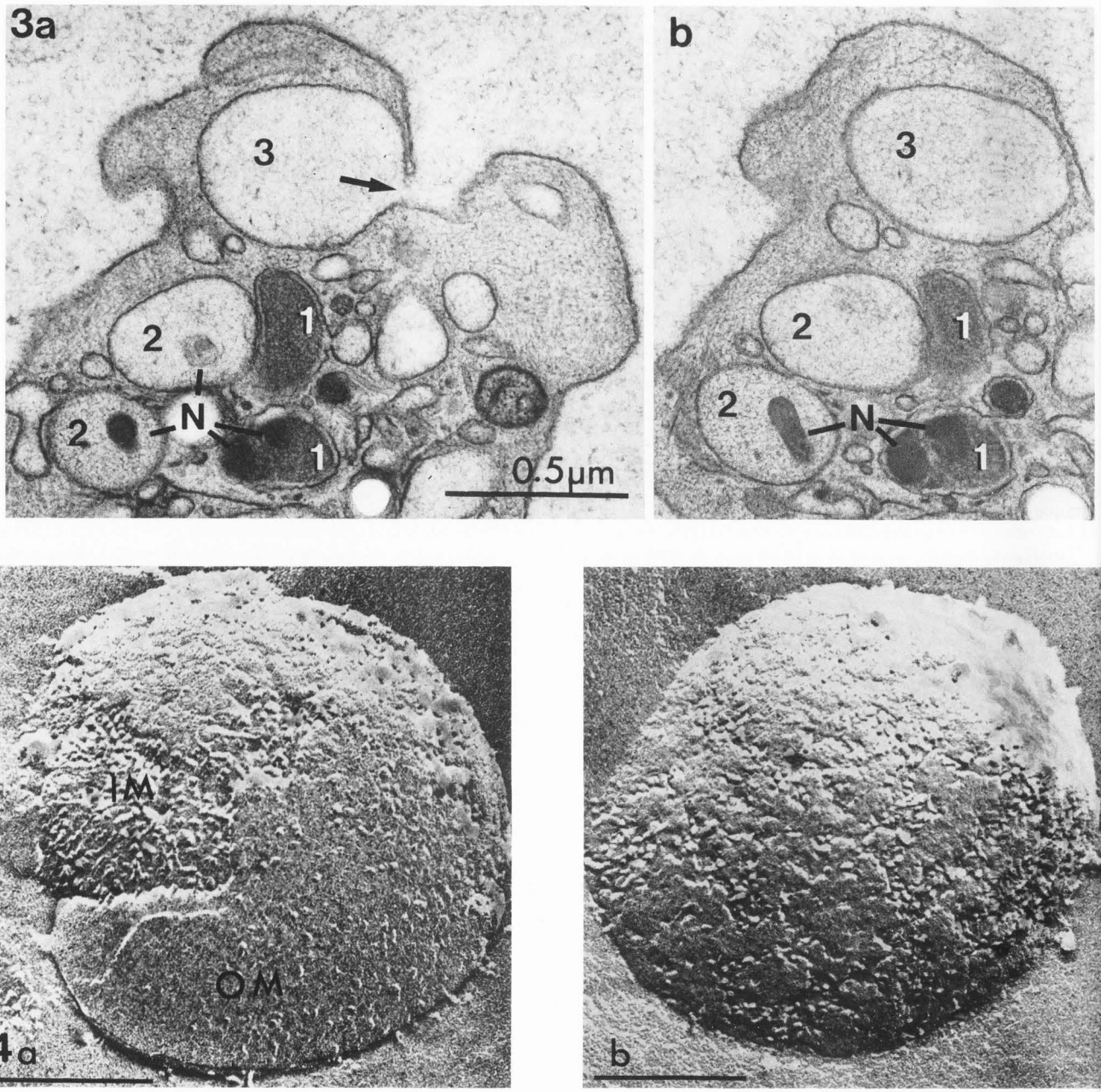

Fig. 3: Freeze substituted human platelets rapidly frozen after stimulation with thrombin. Swelling of the secretory α-granules (originally containing a condensed matrix (1)) with gradual dispersion of the contents ((2), still containing denser inclusions, nucleoids (N)) is obvious. An extensively swollen granule (3) is revealed which has fused with the plasmalemma (arrow in (a)). The small pore (50 nm in diameter) is only visible in this section plane. In the adjacent sections (one of them is shown in (b)) the organelle is surrounded by the organelle membrane and the plasmalemma.
Bar = 0.5 µm. (Micrograph provided by E. Morgenstern, see Morgenstern et al. 1987 for details)

Fig. 4: Freeze fractured isolated mitochondria rapidly frozen during oxidative phosphorylation reveal varying degrees of apposition of outer (OM) and inner (IM) membranes as indicated by the fracture plane deflections between the two membranes. The low degree of membrane interactions in (a) is due to uncoupling, whereas the high degree of interactions in (b) is characteristic for well coupled mitochondria.
(Bar = 0.2 µm. From: Klug et al. 1984, with permission of the publisher)

the functional state of isolated mitochondria, were also shown by EM-work after chemical fixation (Hackenbrock 1966). But only rapid freezing of mitochondria at defined time-points allowed for resolving the kinetics of these transitions (Lang and Bronk 1978). Isolated mitochondria rapidly frozen in different functional states reveal an additional dynamic structural feature, i.e, the variable interactions of the boundary membranes (Knoll and Brdiczka 1983; see Fig. 4). These dynamic contacts depend on metabolic conditions (Klug et al. 1984) and are suggested to be important for regulated metabolite transport (Brdiczka et al. 1985, Riesinger et al. 1985), import of proteins (Schleyer and Neupert 1985) and transport of lipids (Van Venetie and Verkleij 1982). Semifusions of the membranes involving non-bilayer lipids are assumed to represent the molecular basis of these contacts (Van Venetie and Verkleij 1982, Verkleij and Knoll 1986). Rapidly frozen chloroplasts reveal similar structures (Cline et al. 1985).

Processes of moderate speed

Exocytosis-coupled endocytosis. Mode and speed of exo-endocytosis coupling have been found to differ depending on the analysis method. As to nerve terminals, discrepancies depending on the sample layer actually analyzed after fast freezing (Torri-Tarelli et al. 1985) have been discussed above. With Paramecium cells exo-endocytosis coupling is also quite rapid (50 milliseconds for the individual event; 1 s for all events in a cell) but they could conveniently be followed by fast freezing techniques (Plattner 1987a). With a typical gland cell, these phenomena are non-synchronous, and, thus, even more difficult to judge. There are indeed considerable discrepancies in the literature on this aspect. For instance, with adrenal chromaffin cells it was found that antigens located on the luminal side of secretory granules are accessible to antibodies for a very long time (Dowd et al. 1983), while exocytosis-coupled fluid phase peroxidase endocytosis went on much more rapidly (Grafenstein et al. 1986); after tannic acid staining, ultrathin sections also revealed the immediate formation of coated pits indicative of rapid membrane retrieval (Geisow et al. 1985). (For the possible influence of IgG-labeling, also see below). This calls for "rapid" methods which in turn, however, face the problem that freeze-fracturing can differentiate between exo- and endocytosis only on a statistical level (in conjunction with measurements, e.g., of catecholamine output; Schmidt et al. 1983). It would now appear feasible to perform precise kinetic analyses with the help of freeze--substitution methods, eventually combined with markers (to monitor early fusions by EM staining of secretory contents). The methodical potential would now be available for such analyses.

Transcytosis. Impressive evidence of transcytosis has been obtained with the use of peroxidase-tagged antibodies, when they are transported through small intestinal epithelial cells (Rodenwald 1980), or with microperoxidase molecules added to endothelial cells (Simionescu et al. 1975). The latter have only little peroxidatic activity at physiological pH (Plattner et al. 1977) and perhaps for this reason do not damage these sensitive cells. This clearly occurs with horseradish peroxidase (Lin and Essner 1986). (For peroxidase as a marker, also see below).

When endothelial cells were fast frozen and freeze-substituted, the vesicle population observed differed from aliquots processed by "standard" chemical fixation (Wagner and Andrews 1985). Similarly no transendothelial channels could be found (Casley-Smith 1981). Again fast freezing methods appear more reliable, since they act so much faster than chemical fixatives.

Microtrabecular structure. Ice crystal formation during freezing or follow-up procedures (see above) are important, when dynamic microzonations of the kind of the microtrabecular lattice (Wolosewick and Porter 1979) have to be analyzed. Microtrabeculae are, thus, difficult to ascertain by freeze-drying or by freeze-substitution (as by Porter and Anderson 1982) or by freeze etching (as by Heuser and Kirschner 1980). Since it would not be visualized by freeze-fracturing at acceptable temperatures, it might even not at all be possible to depict this structure reliably (Miller et al. 1983). The correlation between local mesh size and speed of saltatory movements (Bridgman et al. 1986) could also be accounted for by different degrees of hydration, which then could be considered as an indirect indication of a different cytosolic substructure. The situation is far from satisfactory, since critical point drying has also been shown to induce - reproducible - artifacts in the cytosolic compartment (Ris 1985). Hence, the existence, shape and dynamics of the microtrabecular structure remains open to further investigations.

Some of the processes that take place with "moderate" speed are discussed below in "Pulse label experiments".

Pulse label experiments

When precursors are allowed to be integrated into a labeled product, the dynamic processing of which has to be analyzed, pulse-chase experiments are advisable. When subcellular constituents are indirectly marked (e.g., by immunocytochemistry), pulse labeling or "permanent" labeling under stationary conditions (see below) can be used.

Autoradiography. Our understanding of transcellular transport phenomena is essentially based upon autoradiographic pulse-chase experiments (Palade 1975). Radioactive labeling is not hampered by the binding of the ligand-marker complexes (see below). A particularly elegant approach is the labeling of living cells by surface iodination (Muller and Gimbrone 1986) or by glycosyltransferases (Schwarz and Thilo 1983). However, radiation spread entails very restricted resolution. Autoradiographic experiments on transcellular transport can now be made with a refined computer simulation of possible radiation sources in a cell (Salpeter and Farquhar 1981). Along these lines further progress has been made by digitizing EM autoradiographic micrographs (Miller et al. 1985). Also the use of physical developers has been shown to yield better resolution than regular processing (Kopriwa et al. 1984). High resolution can also

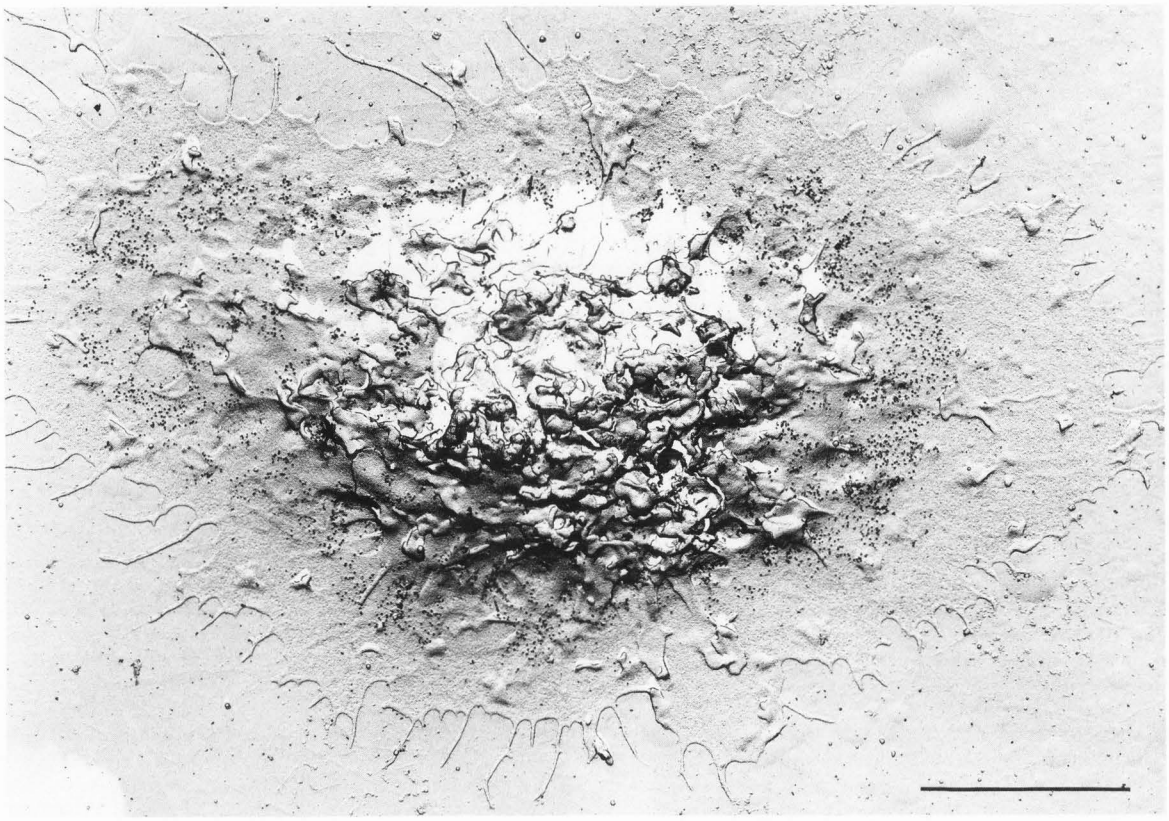

Fig. 5: Surface replica of a mouse peritoneal macrophage grown on a culture dish. The distribution of 40 nm-gold conjugated to acetylated low density lipoprotein reveals a preferential binding to restricted regions of the plasma membrane surface.
(Bar = 5 µm. From: Robenek and Schmitz 1985, with permission of the authors and of the publisher)

be achieved with surface ligand labeling combined with freeze-fracturing (Carpentier et al. 1985). Autoradiographic surface labeling can also be followed by superimposing the backscattered electron (BSE) image to the secondary electron signal (Junger and Bachmann 1980).

Special cell surface analyses. For the last mentioned approach, as well as for any other surface labelings, new yttrium aluminium garnet (YAG) BSE-detectors, combined with field emission electron guns, are of high interest, since this provides considerably improved instrumental resolution (Walther et al. 1984). Surface replica techniques combined with gold labeling also represent a powerful tool to analyze cell surface dynamics (Hohenberg et al. 1985). Fig. 5 shows an example for receptor localization in restricted plasma membrane domains (Robenek and Schmitz 1985).

Indirect labelings. Besides the methods discussed above, the same procedures can be used as indicated below for steady state conditions. However, the time scale (and, hence, the direction of movements) can be strictly determined only when the label is applied as a pulse. Important data had already been obtained with non-specific EM markers. Only later it was recognized that the charge of the marker used, be it peroxidase (Farquhar 1978) or ferritin (Davies et al. 1981), greatly influences the pathway found. Gonatas et al. (1984) faced a similar discrepancy, when they compared wheat germ agglutinin binding, once using ferritin and once peroxidase as a tag. Once again toxic side effects of horseradish peroxidase have to be recalled (Mazariegos and Hand 1985, Lin and Essner 1986). Gold markers of defined sizes (Slot and Geuze 1985) to be used in conjunction with (potentially multiple) immuno- or affinity-labelings would a priori not entail such a problem, because conjugates are fully covered by IgG, protein A, a lectin or another ligand. Yet other methodical problems arose meanwhile in this context. The time required for exo-endocytosis coupling in chromaffin cells appears to be delayed by binding of IgG, as compared to monovalent Fab (Dowd et al. 1983). Similarly Momayezi et al. (1987) could inhibit exocytosis by crosslinking cell surface antigens by IgG, but not by adding Fab fragments. Also in chromaffin cells the formation of coated pits (Geisow et al. 1985) and internalization of a fluid marker (Grafenstein et al. 1986) was much more rapid than expected from IgG labeling studies (Dowd et al. 1983). A marginal remark in the work of Patzak and Winkler (1986) indicates, that the clustering of the label and the pathway of retrieved membranes would not be different when either IgG- or Fab-labeling was used on the EM level. However, their respective effect on the kinetics still

would have to be scrutinized. Indeed such effects have been observed with other cell types (Helenius and Mellman 1983, Ukkonen et al. 1983).

Nevertheless labeling experiments of this kind have successfully explored important aspects of receptor-ligand internalization or their uncoupling and differential targeting (Willingham et al. 1981, Geuze et al. 1984). Recently monoclonal antibodies allowed for a further refinement of such analyses (Dunphy et al. 1985, Weissman et al. 1986). It would be awarding to use Fab fragments derived from monoclonal antibodies for some of the problems discussed in this context. A recent approach for analyzing simultaneous intracellular traffic along different (or the same) routes is the use of double-label cytochemistry as illustrated in Fig. 6 (Hedman et al. 1987). By the use of this method the authors conclude that endocytosis--coupled membrane recycling and exocytosis (which was independent of the endocytosis analysed in the same cells) usually occur in distinct vesicles, but also that some overlap may occur.

Some of the experiments discussed here have not necessarily to be carried out in the pulse--chase mode. They can equally well be done under steady state conditions, when one just tries to find out the pathways involved (see below).

Analysis of dynamic processes under stationary conditions

Molecular level. Methods of image reconstruction can be applied to macromolecular assemblies "fixed" under extreme situations. Negative staining has been applied to isolated gap junctions under conditions of uncoupling (+ calcium) or coupling (- calcium) (Unwin and Ennis 1983). The authors found conformational changes of connexon subunits; calcium causes a twist and, thus, closing of the hydrophilic channels. The limited size of such membrane fragments allowed also for freezing as a thin film and direct observation in a cryo-EM (Unwin and Ennis 1984). Besides the faithful preservation of the native material this preparation method allows for the visualization of the complete structure including the membrane integrated parts of the subunits, which are not shown by negative staining. However, intermediate structures as suggested to exist during the close-open transitions (on the basis of patch-clamp-studies; A. Kolb, pers. commun.), have not yet been shown so far to be accessible with the present methods. Previous freeze-fracture analyses of the overall gap junction structure, following chemical fixation, have been completely invalidated by more recent work using fast freezing techniques (Green and Severs 1984, Miller and Goodenough 1985).

Cryo-EM had also been used to analyze the dynamics of microtubule depolymerization (Mandelkow and Mandelkow 1985), indicating a simultaneous removal of monomers from the ends and from the body of the microtubule. The preservation (no flattening as occurring with negative staining) allowed for a detailed structural evaluation by Fourier transformation. Results thus obtained are well comparable to the data obtained by x-ray diffraction of hydrated bundles before freezing (Mandelkow and Mandelkow 1985).

Another structure analyzed by direct imaging is, e.g., ciliary dynein (Heuser 1981). Fast freezing was used here, however, only to allow for deep etching studies, while the structures had been fixed under working or relaxation conditions and washed with a volatile solvent. In a similar manner myosin crossbridges in muscle actomyosin and coated pit formation could be visualized (Heuser 1981). Similar analyses showing the occurrence and time course of these dynamic phenomena in vivo would deserve great interest.

Another problem to be investigated in more detail is the occurrence of particle movements across the plane of the membrane, as observed in response to local anaesthetics (Sekiya and Nozawa 1983). Furthermore, in response to a trigger for neurotransmitter release, a reduction of membrane particles has also been reported to occur (Israel et al. 1981). The dynamics and the underlying mechanisms have not yet been analyzed.

Cellular level. The label is principally applied (a) as a pulse chase, (b) as a permanent label before fixation ("pre-embedding methods"), or (c) after fixation and further processing ("post-embedding methods"). Methods used are, e.g., labeling with antibodies (or Fab fragments thereof), lectins or other specific ligands (enzymes, activators, hormones, inhibitors, toxins, etc.) Only a brief outline with the focus on current problems and developments can be presented here. For a general survey of immuno- and affinity-EM labeling techniques, see Plattner and Zingsheim (1983).

Colloidal gold particles of defined size (Slot and Geuze 1985, Van Bergen en Henegouwen and Leunissen 1986) allow for simultaneous double labelings, when they are coated with antibodies, protein A, lectins or other ligands (Roth et al. 1981, Handley and Chien 1987). Further progress with regard to resolution, antigenic specificity and capacity may develop from the possibility to covalently link very small gold clusters to specific residues of Fab-fragments (Hainfeld 1987).

The introduction of new low temperature embedding procedures (Lowicryl® resins) has greatly improved sensitivity for intracellular labeling on plastic sections (Armbruster and Kellenberger 1986). Recent achievements with techniques of this kind are the use of transferase-coated gold particles to localize their site of action in the Golgi apparatus (Lucocq. et al. 1987) or the localization of the site of prohormone -> hormone conversion by monoclonal antibodies (Orci et al. 1985).

Comparable analyses on the microzonation of Golgi elements (see Griffiths and Simons 1986) could be made with cryosections from sucrose impregnated aldehyde fixed cells (Tokuyasu 1983).

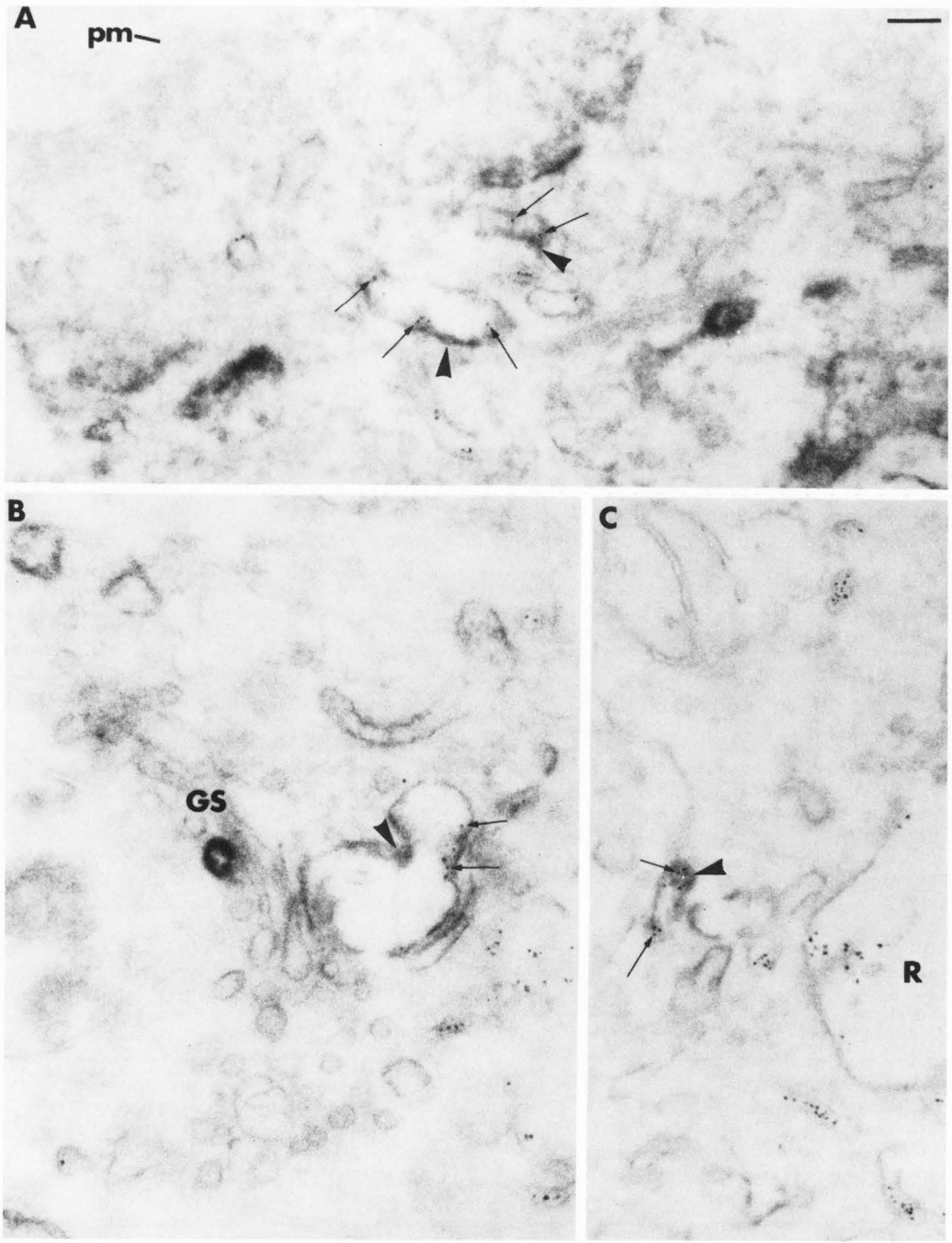

Fig. 6: Double-label cytochemistry. Human epidermoid carcinoma (KB) cells, infected with vesicular stomatitis virus (VSV), show transferrin(TF)-ferritin label (arrows) and immunoperoxidase label for VSV-"G" protein (arrowheads). This figure shows the unusual image of single vesicular structures that contain both TF-ferritin and VSV-"G" protein. The same procedures revealed usually specific label of distinct vesicles. (a): Elongated tubular elements near the plasma membrane (pm), (b): vacuolar structure near the Golgi stacks (GS), (c): vesicular element near a receptosome (R).
Bar = 0.1 µm. (From: Hedman et al. 1987, with permission of the authors and of the publisher)

Problems characteristic for immunolabeling on cryosections and strategies to overcome them are discussed in detail by Boonstra et al. (1987).

Cryostat sections can be infiltrated with peroxidase-labeled antibodies (Brown and Farquhar 1984) to localize sorting of lysosomal enzymes, etc. These authors also indicate precautions to avoid the notorious redistribution of reaction product from diaminobenzidine (Courtoy et al. 1983). Another example is the localization of acidic compartments by a permeable indicator that also serves as a hapten to be localized by antibody-peroxidase conjugates (Anderson et al. 1984).

A very promising approach seems to be the combination of rapid freezing, freeze-substitution and low temperature embedding (Humbel and Müller 1985). Recently uranyl fixatives were shown to preserve well antibody binding capacity (Erickson et al. 1985) and, thus, would lend themselves for combination of these methods with immunocytochemistry. This now also appears possible even without any fixatives, when one processes fast frozen materials with new low temperature embedding media (Carlemalm et al. 1985). This appears particularly important with regard to the localization of reversibly structure-bound soluble antigens. In this case, however, one has to face the possible problem of redistribution of non-fixed cellular constituents during preparation (H. Schwarz, pers. commun.).

Steady state conditions are also given when stationary ion distributions are analyzed by x-ray microanalysis. Some aspects, particularly concerning cryofixation and calcium localization, are discussed above. Zierold et al. (1984) appreciate closed sandwich samples (rather than "slam freezing" on a cold metal block, using "open" samples with an uncontrolled fluid covering, that partly might evaporate as the sample is slammed down), since sandwich freezing (see Plattner and Knoll 1984) allows one to freeze undisturbed cell monolayer cultures in their own medium, in a defined space and composition, without affecting ion distributions. Of course, cells should be kept only for as small a time period as possible within such a closed sandwich; (for vitality tests, see Plattner and Bachmann 1982).

Slow processes

The advantage for the use of cryotechniques even for the analysis of slow processes is exemplified by ameboid movement. Differences in ion concentrations are detected in pseudopodia and other sites of the cells during the slow movement (Schäfer et al. 1985).

It is surprising to what extent cell shape and slow cellular processes can be distorted by chemical fixation. For instance, a goblet cell no more deserves its name, since it has been visualized by fast-freezing and freeze-substitution (Sandoz et al. 1985b). A long time ago the same methods had proved useful to determine the precise width of intercellular spaces in brain tissue (Van Harreveld et al. 1965) and only these data precisely matched the physiological givings. Neuritic growth cones also can be faithfully analyzed only with rapid freezing techniques (Rees and Reese 1981), as was shown for the true morphology of lysosomes (Robinson et al. 1986).

Microtubules are another system quite sensitive to chemical fixation at ambient temperature. Their number is much greater, when cells are fast frozen and freeze-substituted (Sandoz et al. 1985a). The reason might be the considerable turnover between monomeric tubulin and microtubules (Schulze and Kirschner 1987), which could allow for rapid artifacts during chemical fixation at ambient temperature. A "stabilizing" fixative, with guanosinetriphosphate, a Ca-chelator and Mg-ions added (Luftig et al. 1977), later turned out to induce - as a supravital artifact - the formation of new microtubules (Mesland and Spiele 1984) that otherwise would not occur. These multiple contradictory effects make it difficult to establish the "true" appearance of the microtubular system on the EM level and data obtained on the light microscope level, by intravital observations, might sometimes be even more reliable (Schulze and Kirschner 1987).

All these aspects concerning slow processes or even the mere preservation of "static", but sensitive, structures are intimately connected with problems discussed in the following section.

Preparation Artifacts and Appropriate Controls

"Standard" techniques for chemical fixation frequently entail serious distortions of dynamic (and static) ultrastructural details. The fixative action may be too slow (Coetzee and VanDerMerwe 1985) to avoid vesiculation of compartments during chemical fixation (Buckley 1973, Mersey and McCulley 1978, Fernandez and Staehelin 1985). At the cell periphery lamellar structures, thought to contribute to nerve growth cone formation, disappear during chemical fixation (Cheng and Reese 1985). Small vesicles located beneath the cell membrane may totally disappear from fibroblasts (Bretscher and Whytock 1977). Other examples concerning secretory organelles or endothelial vesicles, microtubules or microtrabeculae are discussed above. The book by Rash and Hudson (1979) contains an ample collection of artifacts (induced by chemical treatments) which one could overcome since the sixties by using one out of an ever growing number of fast freezing techniques (see Van Harreveld et al. 1965, Plattner et al. 1973, Plattner and Bachmann 1982, Robards and Sleytr 1985, Gilkey and Staehelin 1986, Menco 1986 and Sitte et al. 1987).

To control any possible effect of the manipulations required for fast freezing techniques, vitality of the cells has to be ascertained, but only before freezing (Plattner et al. 1973). Other controls are provided by in vivo affinity labeling, eventually combined with image intensification which recently has been greatly improved by computer assistance (Allen and Allen 1982, Kukulies et al. 1985). For this microinjection is required in most cases. Microinjected antibody-coated gold particles could first be localized in ultrathin sections (Wehland and

Willingham 1983), but now video-enhanced contrast procedures also allow for identifying gold labeled structures in vivo (DeBrabander et al. 1986). So potentially both levels of observation could now be combined.

However, one has to caution that the application of antibodies to living cells by microinjection also raises new problems, such as redistribution of reaction products (with peroxidase labeling; Momayezi et al. 1986) or crosslinking and sequestration of soluble antigens (unpubl. observ.).

Conclusions

Electron microscopy has substantially contributed to an understanding of cell dynamics. The arrangement of actomyosin elements in muscle fibers had led Huxley (1963) to postulate the sliding filament hypothesis. Meanwhile one can even visualize binding sites of monoclonal antibodies on individual myosin molecules and simultaneously pinpoint interactions with the contraction mechanism (Flicker et al. 1985). A similar progress has been made with regard to exocytosis (Palade 1975) and endocytosis (Geuze et al. 1984, Willingham et al. 1981). The importance of the development of new techniques for deeper insight into problems of cell dynamics can be easily judged, when these data are compared with the first visualization of the same processes by DeRobertis and Vaz Ferreira (1957) and by Palade (1959). These processes can now also be faithfully analyzed in the millisecond frame (Plattner 1987b). Most impressive are recent data on routing of different ligands and receptors from the cell surface to the interior (Willingham et al. 1981, Geuze et al. 1984) and on the sequestration phenomena occurring in the Golgi area (Griffiths and Simons 1986).

All the progress achieved has depended on the development of methods and instrumentation. It remains to be hoped that this critical balance of current achievements, goals, problems and perspectives favours discussions on new developments.

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Discussion with Reviewers

BA Afzelius: You make it clear that the only useful preparation technique that is able to preserve fast and very fast processes are such that involve various quick-freezing methods. An alternative method would have the advantage that it is even quicker than the quickest freezing method and one that penetrates at a great depth, is the use of a strong neutron flash. This has been suggested and evidently also attempted successfully by John Luft from University of Washington in Seattle. Do you have any comments?

Authors: Cryofixation has the limitations given by the physical properties of water: poor heat conductivity restricts the efficiency near the surface of even optimally cooled biological materials. Any alternative that would help us to overcome these limitations would be revolutionary and open up new fields. But applicability of alternatives with a similar possible impact still would have to be shown.

BA Afzelius: The first fixation method that was quick enough to preserve the cilia of protozoa in their metachronal waveform was the chemical fixation technique used by Parducz (1967). He used a mixture of strong osmium tetroxide and mercuric chloride at room temperature or above. This technique evidently is so quick that it can do what some of the early freezing methods could not. Do you have any information of whether other chemical agents, such as acrolein, could be quick enough to compete with the freezing methods, and whether the quickest fixation methods in these chemical alternatives would be used at an increased temperature.

Authors: Rapid freezing techniques are grown up considerably since these early days. What has been pointed out above as a principal drawback of the freezing approach, the poor heat conductivity of ice, has an equivalent for chemical fixatives, i.e., the limitations given by diffusion velocities. We believe that dissipation of heat should be much more rapid than diffusion of chemicals. The problem is emphasized by the difficulties which are especially obvious with plant cells due to their very poor diffusion properties.

K-R Peters: Which techniques are available to image in bulk, quick frozen specimens, molecular fine structures without having to warm the specimen up above 140 K?

Authors: The real problem is to freeze bulky specimens quick enough (of course without cryoprotectants). Only then the avoidance of higher temperatures (> 140 K) would be of any significant advantage. If this would be possible, freeze fracture (but not etching) under very good vacuum conditions would be possible.

K-R Peters: Do you know about any possibilities for freeze substitution and embedding/etching drying below 140 K?

Authors: No. Data available so far point to applicability of these techniques only above that critical temperature range. For etching, see above.