

**PATTERN ANALYSIS OF GAP JUNCTION PLAQUES
WITH OPEN AND CLOSED PORES**

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The structure of freeze-fractured gap junctions was studied by electron microscopy and subsequent pattern analysis using a computer controlled image processing system. Rat mammary tumor cells (BICR/MIR-k) which are permanently coupled via gap junctions when cultured as monolayers were used under different fixed and unfixed conditions. Active (coupling competent) gap junctions seem to be characterized by loosely packed connexons, whereas non-active (permanently closed) gap junctions may consist of tightly packed particles.

Introduction

Information can be exchanged between cells by different mechanisms such as hormonal interaction, neuronal signal transfer or direct intercellular communication. In embryonic development, this direct junctional cell to cell diffusion of small molecules is the first information transfer system. In fully developed organisms, direct intercellular communication plays an important role for regulation processes in organs and tissues whereas a disturbed communication may cause malignant growth /6/. The membrane channels which enable the passage of molecules from one cell to its attached neighbours are always aggregated to plaques which may consist of up to several hundred individual channels: the gap junction. A single gap junction channel consists of two hemi-channels, the connexons which are provided by each of the communicating

cells. Structural data provide evidence, that a connexon is a hexamer of intramembranous proteins, the so-called connexins /4/. The gap junctional contact is characterized by a 3 nm gap between adjacent cells.

Under the electron microscope, gap junction plaques can be visualized either in thin sections of embedded cells or by freeze fracturing the cells. In replicas of freeze-fractured gap junctions of vertebrate cells the proteins remain in the p-face-leaflet of the membrane whereas the corresponding pits are seen on the e-face-leaflet /3/ as is demonstrated in FIG. 1.

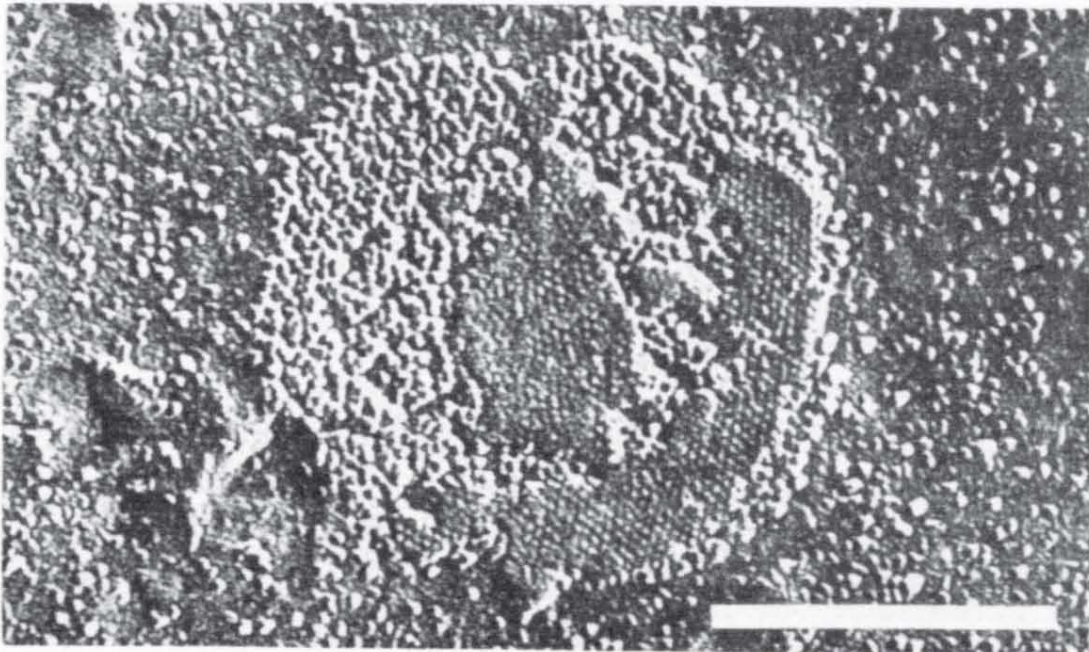


FIG.1 Freeze-fractured gap junction between conventionally fixed BICR/M1R-k monolayer-cells with p-face (left) and e-face (right) areas. Bar: 0.25 μ m

Cells can be either in a coupled or in an uncoupled state, depending on functional activities, which are regulated by pH, Ca^{++} , cAMP or transjunctional voltage /11/. The question whether the morphological appearance of gap junctions in freeze-fracture replicas can be correlated with the physiological state of the cells is still open. Several investigations dealing with this problem based on glutaraldehyde fixed cells /1,2,7,8,9/.

Our electrophysiological experiments showed, however, that glutaraldehyde rapidly uncouples intercellular communication, confirming results reported by Berdan and Caveney /1/ as well as by Spray et al. /10/. Therefore, we investigated whether glutaraldehyde fixation alters the connexon arrangement in gap junctions and whether their morphological appearance could be correlated with the coupling status of the cells.

Materials and Methods

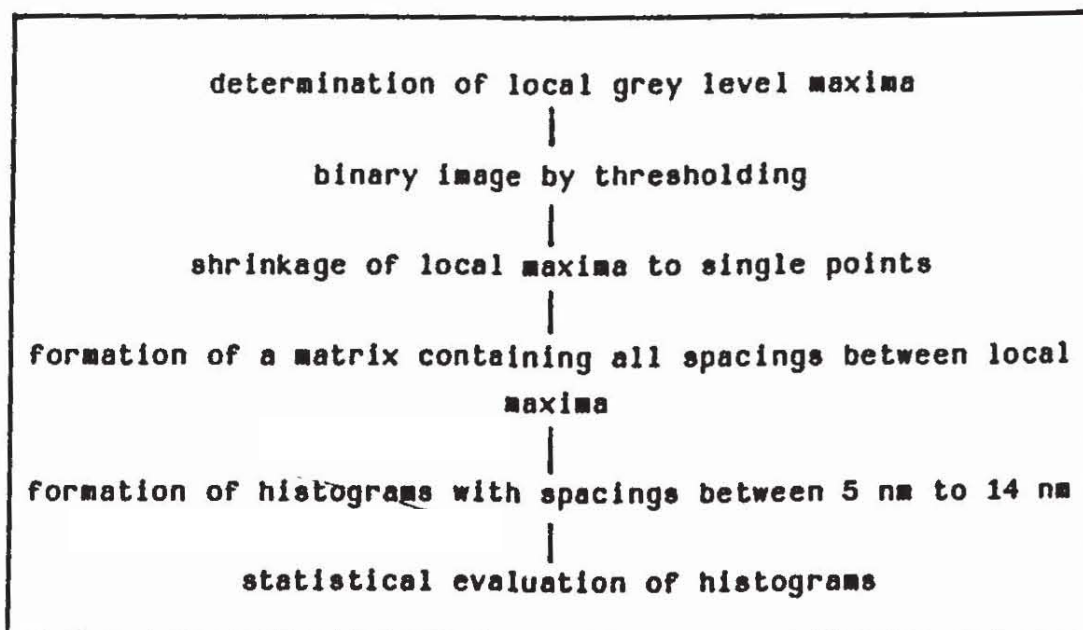
Rat mammary tumor cells (BICR/M1R-k) which are permanently coupled via gap junctions when cultured as monolayers have been investigated /5/. In order to gain information about the effect of glutaraldehyde we used different preparation procedures for these cells before freeze fracturing them at $-150\text{ }^{\circ}\text{C}$ and at a vacuum of $= 2 \times 10^{-6}$ Torr in a Balzers BAF 301 instrument:

- Conventionally fixed cells: 30 min glutaraldehyde fixation, glycerinated for cryoprotection, frozen by freon dip.
- Shortly fixed cells: 5 min glutaraldehyde fixation and immediately frozen by freon dip.
- Unfixed cells: frozen by propane jet.
- Unfixed cells: frozen by freon dip.

Fractured cells were replicated by platinum-carbon-evaporation (45° , 2 nm) and carbon-evaporation (90° , 20 nm). Replicas were cleaned for up to 15 hours in sodium hypochloride and washed 5 x in distilled water. Electron micrographs were taken with a Zeiss EM 10 electron microscope.

For the analysis and quantitative evaluation of gap junction pictures obtained with different preparations a computerized image processing system was used (see TAB 1). The electron microscopical micrograph negatives were digitized by an A/D-converter, resulting in images with a resolution of 256 grey levels. The next operation modes were framing of junctional areas and detection of centers from gap junctional particles or pits by thresholding, skeletonization and other

segmentation techniques. The data were recorded on tape and analyzed further on a VAX computer. The center-to-center spacings between all skeletonization points were measured and classified by 1 nm steps, but only spacings between 5 nm and 14 nm were included for subsequent determination of frequency distributions and statistical analysis. These parameters characterize the gap junction pattern in a computer-applicable mode. In addition nearest neighbour distances were determined from these collected data.



TAB. 1 Scheme of image processing procedure

Results and Discussion

In all four preparation procedures junctional e-faces often exhibited a more regular particle pattern than p-faces, resulting in first order spacings with more distinct peaks, as is shown in FIG. 2 for conventionally fixed cells. Our comparison of the different preparation methods revealed a shift in the frequency distribution to higher values when junctional particles were analyzed in cell preparations which were either unfixed and frozen by propane jet or shortly

fixed and frozen by immersion into freon (FIG. 3). The mean particle distance to nearest neighbour, however, was reduced in unfixed propane jet or shortly fixed preparations. Classification experiments based on measured statistical parameters according to the special features of the gap junction appearances permitted a more detailed interpretation. The computer memorized distinct gap junction classes (as defined by preparation procedure and fracture face of the analyzed replica) by the frequency distributions of their respective particles or pits. Thereafter, unknown gap junctions were attributed to the acquired class parameters (TAB. 2). Class A includes p-face gap junctions of fixed and glycerinated cell preparations, class B accordingly prepared e-face gap junctions.

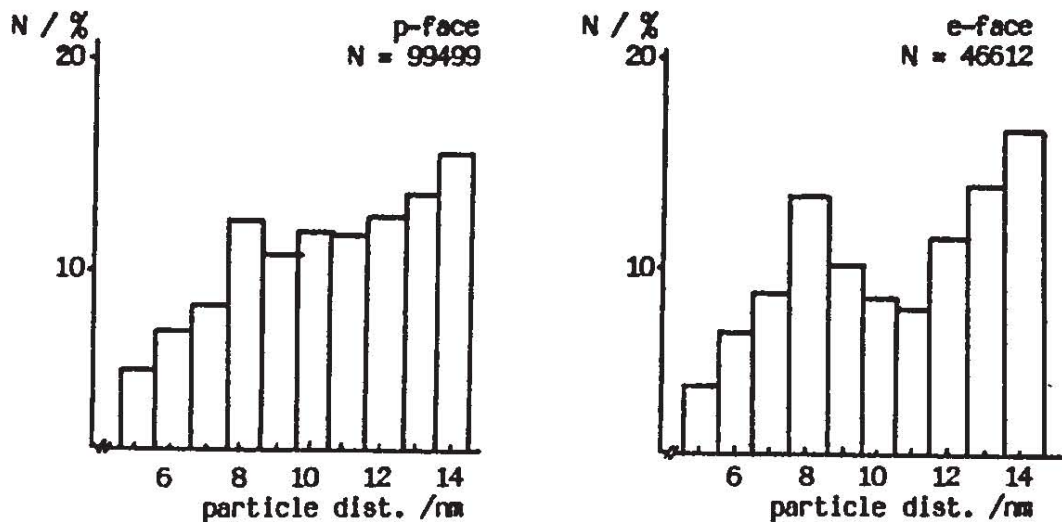


FIG. 2 Frequency distribution of center to center spacings of gap junction particles (p-face) and pits (e-face) of conventionally treated BICR/M1R-k monolayer cells. Mean particle distance to nearest neighbour: 6.85 nm (p-face) and 6.43 nm (e-face).

The relative unequivocal reclassification of class A and class B points at the fact that gap junctions from conventionally treated preparations constitute a very homogeneous class. Interestingly, both preparations exhibit also some class F (loosely packed) gap junctions. However, p-face (class C) and e-face (class D) gap junctions from unfixed cells frozen by immersion into freon are more heterogeneous. Some of these gap junctions resemble those of fixed cells, whereas others are clearly different, with clustered or loosely arranged particles. Other examples for heterogeneity are class E which represents unfixed gap junctions frozen by propane jet and class F which consists of shortly fixed gap junctions frozen by immersion into freon. We conclude from our electron microscopical investigations and computerized analysis of gap junction micrographs that glutaraldehyde fixation and glycerination alters the morphology of gap junctions. Unfixed cells frozen either by immersion into freon or by propane jet exhibit a heterogeneous appearance of gap junctions, whereas in conventionally fixed preparations mainly tight particle configurations are displayed. In addition, gap junctions of shortly fixed cells form clusters which may represent an intermediate stage of the aggregation following a glutaraldehyde fixation.

The tight particle packing may reflect the non-active state of the gap junction, when all pores are permanently closed. Active gap junctions are characterized by loosely packed particles, they may open or close their pores accordingly to functional necessities. Both active and non-active gap junction plaques can be present in BICR/M1R-k monolayer cells under normal conditions but they are distinguishable only in shortly fixed or unfixed cell preparations.

Up to now the question whether glutaraldehyde uncouples cells by direct action on gap junction proteins or by elevation of intracellular Ca^{++} is still open. Since glutaraldehyde causes a rapid uncoupling of cells, an investigation of open gap junctions is prevented by this fixation method. Glutaraldehyde fixation, however, entails an alteration of

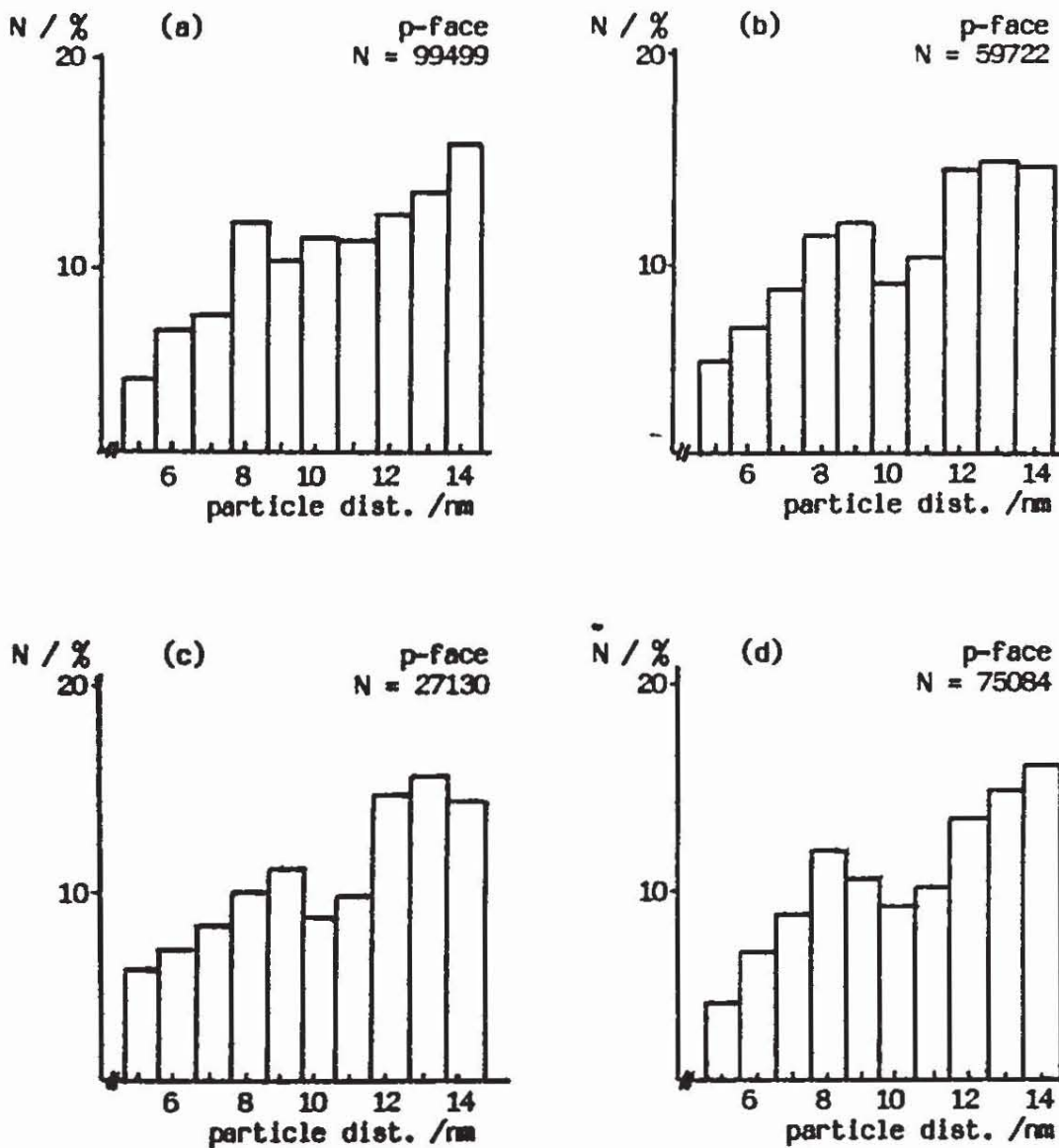


FIG. 3 Frequency distributions of center to center spacings of gap junction particles (p-face) of BICR/M1R-k monolayer cells and mean particle distance to nearest neighbour.

- a) conventionally fixed (6.85 nm)
- b) shortly fixed (6.43 nm)
- c) unfixed propane jet (6.52 nm)
- d) unfixed freon dip (6.82 nm)

gap junctional protein arrangement only after a longer (> 30 min) period, therefore, shortly (5 min) fixed cell preparations are still very helpful in distinguishing active from non-active gap junction plaques.

defined class	computer classified class					
	A	B	C	D	E	F
A	36	1	0	0	0	4
B	2	24	0	0	0	5
C	11	4	16	0	0	5
D	0	2	1	0	0	1
E	1	0	1	0	0	5
F	7	0	1	0	0	16

TAB. 2 Classification of different gap junction preparations

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