COMPLEMENTARY REPLICATION OF GAP JUNCTIONS IN SHEEP CARDIAC PURKINJE STRANDS: EVIDENCE FOR NONCOMPLEMENTARITY OF PARTICLES AND PITS

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SUMMARY

P-face particles and E-face pits of freeze-fractured gap junctions are widely considered to be complementary structures. In the present work, complementary replicas of gap junctions in freeze fractured sheep cardiac Purkinje strands were studied using a new method for matching complementary features in the electron micrographs of complementary replicas by superimposing their stereo images. When a stereo image of a defined area of E-face is superimposed on the stereo image of the corresponding area of P-face, the pits fall between the particles, not on them. It is concluded that E-face pits and P-face particles are noncomplementary.

Key Words:

Freeze-fracturing

Gap junctions

Junctional structures

Purkinje cells

Membrane proteins

Species: Sheep

Electron micrographs of freeze-fractured tissues containing gap junctions (GJ) show characteristic patterns of more or less regularly arranged particles and pits on the P- and E-faces of the fractured membrane (Peracchia, 1980). Current concepts of GJ structure regard the particles and pits as complementary structures which result from the splitting of the connexon, an assembly of integral membrane proteins made up of six identical polypeptide subunits surrounding a central channel (Makowski, et al., 1977, 1984). The interpretation of P-face particles and E-face pits as complementary implies that in the native (in situ) state, the structures from which these two components originated lay on the same transmembrane axis, and that the transmembrane axes of the particles and pits are identical and correspond to the locations of the cell-to-cell channel. It is widely recognized that the E-face pits of GJ are more closely spaced and more highly ordered than the P-face particles (Peracchia, 1980). These discrepancies are reconciled with the supposed complementarity of particles and pits by postulating that distortion of the native connexon array by "plastic deformation" during freeze-fracture affects the P-face particles more than their complementary E-face pits (Peracchia, 1980).

Complementary replicas of GJ investigated recently in freeze-fractured Purkinje strands from sheep hearts exhibit structural characteristics inconsistent with the notion that their particles and pits are complementary. In the present paper a new method of matching complementary features is described. It involves superimposing stereo images of the complementary replicas. When the stereo image of a defined area of E-face is superimposed on the stereo image of the corresponding area of P-face, the pits fall between the particles, not on them.

An abstract of this work has been previously published (Kordylewski and Page, 1985).

MATERIALS AND METHODS

Sheep hearts were obtained immediately after slaughter of the animals. Still at the slaughterhouse, Purkinje strands were quickly excised from the ventricular chambers and immediately immersed in 1.5% glutaraldehyde buffered to pH 7.4 by 150 mM Na cacodylate. The specimens remained in the fixative for one hour at 4° C, during which they were transported to the laboratory. The Purkinje strands were then glycerinated and freeze-fractured with unidirectional shadowing on a Balzers BAF 301 apparatus and further processed as described by Kordylewski, et al. (1983, 1985), except that the Balzers complementary replication device replaced the conventional specimen holder. The replicas were cleaned with Chlorox and distilled water, collected on 300 or 400 mesh uncoated finder grids and photographed at original magnifications of X 50,000 to X 200,000 in a Hitachi H-600 electron microscope equipped with a eucentric, side-entry goniometer stage and a Hitachi H 5001M multispecimen holder that permitted both alternate viewing of complementary replicas and tilting of the specimens. Stereo pairs were photographed at tilt angles of 5° with respect to each other.

Techniques for comparing and superimposing complementary E- and P-faces of the same gap junction: Multiple techniques for investigating the complementarity of E-face pits and P-face particles in complementary replicas of the same junction have been developed. The application of each technique was preceded

by transilluminating on a light box the negative films of stereo pairs of electron micrographs of both complementary fracture faces photographed at original magnifications of X 50,000, X 100,000, or X 200,000, and inspecting them with a stereo viewer (magnification X 2) to yield a final magnification of X 100,000, X 200,000, or X 400,000. In applying the additional techniques described below, such stereo views of the areas under study (like those in Figures 4, 6, and 8) were always at hand, so that the three-dimensional structure could be recognized and the landmarks of the gap junctional area could be identified. By insisting on stereo views before applying other techniques, the areas least distorted by tilt or non-planarities of the surface could be selected. Furthermore, the preliminary stereo view was essential for aligning and then matching details of the E- and P-faces superimposed by various techniques with respect to external landmarks (most commonly either caveolar necks on the non-junctional plasma membrane cross-fracture edges, or the boundaries of the gap junctional plaques). The structural features of the gap junctions (e.g., unusual arrays of particles, valleys between the particles, particle-free patches, etc.) have also been used as landmarks for matching the complementary details on the other face.

The additional techniques included:

(1) projection of a two-dimensional (non-stereo) image of the E-face onto a reverse-printed photograph of the corresponding area of the P-face (or vice versa);

(2) projection of the negative of an area of P-face particles onto the screen of a Nikon Profile Projector (Model 6C-2) (negatives were enlarged 10- or 20-fold to give final magnification X 10^6), and tracing the particle outlines onto a sheet of tracing paper taped to the screen; the negative of the P-face was then removed and replaced with the negative of

the complementary area containing the E-face pits, aligned with respect to the landmarks as described above, and the locations of the projected pits to the outlines of the particles were determined. Also both negatives were superimposed on each other on the stage of the Nikon Profile Projector and the alignment of the projected images was checked by flipping the upper negative and bringing it in and out of focus until the appropriate alignment was achieved. Then the edges of the negatives were secured by adhesive tape. In this way "double" negatives were made in order to print a middle composite picture in the triplets (Middle picture, Figures 5, 7, 9, and 10). See technique 3 below.

(3) Mounting an array of three contact prints from photographic negatives obtained on the electron microscope (magnifications X 50,000, X 100,000, or X 200,000). The prints were aligned in such a way that each of the outer pictures in the set could be used for viewing as a stereo pair with the middle (composite) picture (Figures 5, 7, 9, and 10). Figure 1 illustrates the four step sequence by which the final desired mounting was obtained. Step 1 shows the complementary replicas of a junction photographed at 0^{o} tilt; the pictures of two complementary replicas have been mounted in a conventional way, i.e., symmetrically, as they would appear if one opened the lipid bilayer like a book. Stereo viewing of both faces was more helpful for understanding the three-dimensional structure of freeze-fractured gap junctions than two-dimensional imaging. Therefore, stereo pictures of each complementary area (E-face and P-face) were routinely made by taking two pictures of the same area at a tilt of 5^{o} with respect to one another (step 2). The stereo pairs of both complementary faces were then examined with a stereo viewer to check their planarity and to determine their three-dimensional

features. The superiority of stereo viewing over regular views of the replicas becomes apparent when the two types of images (planar and stereo) are compared, as in Figures 3 and 4. However, a conventional comparison of pictures of complementary replicas mounted as "mirror images" did not allow sufficiently precise matching of complementary details. In step 3 the negatives of the P-face stereo pair were therefore reversed by revolving them 180° around the long axis of the negative; as a result, the arrays of particles on the P-face stereo pair were parallel to the arrays of pits on the E-face stereo pair. Next, the two middle negatives (the members of each stereo pair photographed at 0° tilt) were superimposed (step 4). For this purpose the matching of landmarks and other details was checked by projecting the two superimposed negatives onto the screen of the Nikon Profile Projector 6C-2 (see above, technique 2). Then (as illustrated in Figure 1), the resulting composite, or "sandwich," containing images of both complementary replicas photographed at 0° tilt, was mounted as the central picture of a three-print array; the left- and right-hand members of the array were, respectively, the -5° and +5° tilt members of the E-face and P-face stereo pairs. The two stereo pairs that can be looked at with a stereo viewer using this array are [A + (B + C)] and [(B + C) + D]. The center picture combines elements of both complementary E- and P-face images. Therefore, switching back and forth between binocular and monocular observations (by alternately obstructing one eye or the other) enables the viewer to obtain the projection of a non-stereo image of the particles onto a three-dimensional image of the corresponding pitted area (the left stereo pair); or the projection of the non-stereo image of the pits onto the three-dimensional image of the particulate area (the right

stereo pair). The arrays of three prints in Figures 5, 7, 9 and 10 should be viewed in this way with the aid of a stereo viewer. Exceptionally, all three prints of the set in Figure 11 were taken at 0^{o} tilt since, when using the high magnification (X 400,000) needed for this array, a stereo effect could not be obtained. Nevertheless, even the naked eye inspection of the three picture arrays in Figures 5, 7, 9, 10, and 11 somewhat shows the relation between the complementary details.

(4) Superimposition of the stereo images of complementary replicas of gap junctional E- and P-faces using polarized light. For this purpose four Eastman Kodak slide projectors were equipped with polarizing filters. Two projectors were used to project aligned stereo pairs of a selected area of P-face onto a lenticular screen which was viewed through polarized glasses (Polarite 3D Viewer, Marks Polarized Corp., Whitestone, N.Y.). Two other projectors were used to project aligned stereo pairs of the E-face from the complementary replica on the same screen, so that (by the landmarks) the P- and E-faces were appropriately aligned. By rapidly alternating the projection between P-face and E-face, it was possible to achieve a "stroboscopic" effect that showed the location of complementary particles and pits relative to one another in three dimensions.

(5) "Mapping" and numbering the gap junctions by inscribing the shapes of gap junctional P-face particles (as visualized in techniques 3 and 4, above) in the appropriate spaces between pits. The 3-M "Sensitron" Model 583 copier produced copies of the prints enlarged 20- to 50-fold. In addition to enlargement, the advantage of these copies was the high degree of contrast of all dark shapes, i.e., of the pits and the shadows of particles. By comparing these high-contrast pictures to the stereo images described in techniques 3 and 4, it was possible to identify each

particle and to determine reliably its location on the fracture face as well as the location of the structure corresponding to it on the complementary fracture face. These particles were then numbered on the photocopied images of the P-face, and the white areas corresponding to each number on the P-face face were labeled with the same number on the complementary image of the E-face (Figure 12).

After making "maps" of the complementary P- and E-faces and numbering them, the E-face map was overlaid with tracing paper and the pits identified on the map were traced and connected by lines with a sharp pencil (Figure 13a and b); the particles on the map of the P-face were similarly traced (Figure 13c). Next the two tracings were combined (Figure 13d) by superimposing them. The shapes of the spaces between pits were used to identify the corresponding particles (after first using tracings of structural features outside the junction for a preliminary orientation of the complementary replicas, as well as stereo viewing the superimposed images as in Figures 5, 7, 9, and 10).

(6) Viewing two (non-stereo) images (negative films) of complementary replicas with the comparator ("blink microscope", Carl Zeiss Jena, Model 1638). This instrument, located at Yerkes Observatory, Williams Bay, Wisconsin, was used by astronomers to compare photographs of the nocturnal sky taken at different times. Although this instrument produces planar rather than stereo images, its very high resolution is exceptionally good. The image thus obtained could be related to that seen by stereo viewing, and could also be used to map particles and pits as in technique 5.

(7) Viewing prints of stereo pairs of complementary replicas with a large mirror stereoscope normally used for analysis of aerial photo-

graphs. The instrument used was in the map collection of the University of Chicago Library. To use this technique, the prints of two stereo pairs were first precisely aligned and superimposed. By rapidly flipping the upper set of stereo pairs, it was then possible to alternate between stereo views of the P-face and the complementary E-face. In this way, corresponding 3-dimensional structural features on the complementary replicas could be reliably identified and their locations on the complementary fracture faces could be compared.

Unlike techniques 3 and 5, techniques 1, 2, 4, 6, and 7 do not yield publishable records.

Measurement of surface densities for P-face and E-face of GJ: The x-y coordinates of centers of particles and pits were digitized from reverse printed photographs at magnifications of X 510,000 - 912,000 using a Ladd Graphic Data Analyzing System; their numbers/μm^2 of membrane fracture face were computed as previously described (Kordylewski, et al., 1983, 1985; Page, et al., 1983).

RESULTS

Model of the gap junction: It is convenient to describe the results of experiments on the spatial relationships of gap junctional particles and pits with reference to the model or "cartoon" in Figure 2. The model was drawn by an artist on the basis of data obtained using the methods described in the Methods section and in the paragraphs to follow. Reference to Figure 2 will facilitate discussion of the structural features in the electron micrographs

of complementary replicas (Figures 3 to 13).

Figure 2 depicts a gap junction that has been split into the two complementary replicas A and B in the process of freeze-fracturing the two cells, C_1 and C_2, which are coupled by the junction. A detailed interpretation of the model is deferred to the Discussion. The model is, however, particularly useful at this preliminary stage to indicate how the component membranes of the gap junction fracture in the observed complementary replicas. The diagram illustrates that the E-face appearing in complementary replicas is always derived from the membrane of the same cell as that from which the complementary P-face originates. As noted by Peracchia (1980), the en face fractures through the plane of the intercellular gap do not occur, hence the gap was not visualized in the complementary replicas. A noteworthy feature of Figure 2 is that in unidirectionally shadowed complementary replicas the E-face pits appear as depressions no matter from which of the two cells making up the junction the E-face is derived. A second noteworthy feature illustrated in the model, one which can readily be confirmed by stereo viewing appropriately mounted electron micrographs as in Figures 4, 6, and 8, is that the E-face areas between the pits appear convex because they are bulged upward by the P-face particles that lie directly behind these areas.

Figure 3 shows the complementary fracture faces of a junction mounted in the "open book" configuration for non-stereo viewing, as in step 1 of Figure 1. This and all other electron micrographs were double printed so that particles appear white, whereas pits and shadows of the particles appear black. The symmetry of the complementary junctional fracture faces is apparent, as is that of the caveolar necks whose complementarity is evident near the top of the picture. In Figure 4, the two complementary replicas of Figure 3 were photographed as stereo pairs of each replica and mounted for

separate stereo viewing of each replica (as in step 2 of Figure 1). This way of mounting and viewing facilitates recognition of the layered structure of the junction and of the way the junction splits into the complementary replicas. In Figure 5, the stereo pairs of the complementary replicas in Figure 4 have been mounted as described in Figure 1 (step 4). This display, as well as similar but somewhat more favorable 3-fold arrays in Figures 7, 9, and 10, demonstrates that the pits on the E-face overlie the spaces between particles on the complementary P-face. Conversely, the pattern of P-face particles can also be traced on the E-face. In the composite central panel (Figure 5) the branched pattern of the E-face seen in the left panel is exactly filled with the particles lying in the same area of the P-face in the right panel. Figures 6 and 7, photographed at the higher magnification of X 10^5, reinforce these conclusions.

Figures 8 - 13 show complementary replicas of a gap junctions which chanced to fracture in such a way that only the pitted E-face appeared on one replica and only the particulate P-face appeared on the other replica; i.e., unlike the complementary replicas of Figures 3 - 7, no patches of E-face are shared by both replicas in Figures 8 - 13. It is nevertheless clear from Figure 8 that a layer of particles is present under the E-face of the lower stereo pair; the evidence is the bulges of the area between the pits by which the underlying particles manifest their presence. Stereo viewing of Figures 9 and 10 supports this conclusion by showing that the pits fall between particles, not on them. At very high magnification (X 400,000) the conclusion is also suggested even without stereo viewing (Figure 11).

Figure 12 illustrates the application of technique 5 in the Methods section to the right and left panels of Figure 11. The figure also show that the configuration of the spaces between E-face pits often defines the shape of

the particles (or aggregates of particles) that fall between the pits on the corresponding complementary P-face. In Figure 13, a system of coordinates (a) is defined by lines connecting the E-face pits (b); the particles from the complementary replica (c) are shown to superimpose on the spaces between the coordinate lines (d).

Both the use of the Zeiss "blink microscope" (technique 6) and of the large mirror stereoscope (technique 7) confirmed the structural conclusions reached on the basis of the above-described analysis.

Comparison of numbers of particles and pits for equal areas of gap junctional P- and E-faces: Table 1 gives the values (in number per μm^2) for particles and pits measured in corresponding areas of complementary replicas. The sample consisted of the four gap junctions from sheep cardiac Purkinje strands. Mean center-to-center nearest neighbor distances (NND), calculated from the digitized x-y coordinates of particle centers and pit centers, are also given. The table shows that the number of E-face pits exceeded the number of P-face particles in all four junctions by a factor of 1.34 to 1.51. At the same time, NND was consistently smaller for pits than for particles.

DISCUSSION

Origin and critique of the idea the GJ particles and pits are complementary: The notion that GJ particles and pits are complementary structures is widely accepted, as is the corollary that both lie on the axis of the cell-to-cell channel (e.g., see Peracchia, 1980, fig. 5E). This concept does not, however,

rest on extensive studies of complementary replicas. The question has not previously been examined with stereo imaging of complementary replicas, followed by superposition of carefully aligned images of the complementary fracture faces, i.e., with the techniques that are found essential for demonstrating non-complementarity.

Even when complementary replication was attempted, the usual procedure has been limited to "mirror image" photographs of the complementary fracture faces (Chalcroft and Bullivant, 1970; Steere and Sommer, 1972). The idea that pits and particles might be non-complementary was considered in mouse liver gap junctions freeze-fractured in situ by Goodenough and Revel (1970), who stated that "close examination of the serrated edge between the two views of the junctional membranes suggests that the pits correspond to some of the spaces between the particles and not to the center of the particles themselves. In favorable views...the pits closest to the serrated edge are seen in line with the spaces between the immediately adjacent particles of the particulate lattice....The data available at present do not indicate that the pits are openings of channels passing through the junctional membranes, but it is not possible to eliminate this suggestion."

Caspar et al. (1977) studied freeze-fractured (in situ) mouse liver gap junctions in which the particle pattern was rendered more highly ordered by perfusing the livers with 0.5 M sucrose before fixing them. They found that the pattern of E-face pits could be approximately matched with (superposed on) the P-face particles, and concluded that "the order in the two faces is similar." They did not comment on the issue of complementarity of particles and pits. Their result does not prove complementarity of particles and pits; for example, appropriate translations of the pit pattern along the x- and y-axes would shift their location to the spaces between particles, leading the

observer to infer non-complementarity. Examination of the regular "crystalline" arrays of pits and particles may therefore result in ambiguous conclusions. Instead, in the present paper, irregular patterns of pits and particles to serve as landmarks that could be unequivocally matched on both faces were deliberately sought.

Based on the assumption that, in the native state of the junctions, the structures corresponding to GJ particles and pits lie on the same transmembrane axis as the cell-to-cell channel, the finding that particles are more widely spaced and less orderly than pits in freeze-fractured junctions has been attributed to plastic deformation during freeze-cleaving. This process has been thought to affect the particles more than the pits (Peracchia, 1980). Persuasive evidence does indeed exist for plastic deformation of membranes and proteins during freeze-cleaving (Bullivant, 1974; Sleytr and Robards, 1977). That plastic deformation may occur does not, however, explain the systematic relationship between particles and pits described at present -- pits falling between particles, particles bulging behind the spaces between pits.

Apparent exceptions to this systematic relationship merit discussion. The model in Figure 2 predicts a 1:1 ratio of pits to particles, but it has been found that, in complementary areas of complementary replicas, the number of pits significantly exceed the number of particles. Three artifacts have been identified that may contribute to a spuriously low particle count without affecting the count of pits: (a) some particles are lost during freeze-fracture for unknown reasons; (b) two or more particles may appear to be fused and therefore be counted as a single particle, and (c) some particles may cast shadows that obscure other particles. Moreover, excessively heavy shadowing may obscure the pits. This artifact is usually readily identifiable in some

areas in which the topography of the replica varies; consequently, the slope of the E-face is oriented unfavorably with respect to the angle of shadowing and the pits do not show (e.g., Figure 6).

Nature of the pits: What are the pits? The present data indicate that they are neither, as previously thought, concavities or depressions left by pulling the particles out of the E-face. The focus of the inquiry about their nature must be on the material in the "spaces" between particles. In the model of Figure 2, the areas between the pits are seen to be tented upward, a convexity caused by the particle bulging into the back of the E-face membrane. The bulge corresponds exactly in location to its mate (the other P-face particle of the same connexon) on the complementary P-face. Shadowing reveals that the pits are the points of deepest depression in the area surrounding the particles hidden behind and bulging into the back of the E-face. A different way to express the same idea is to regard the E-face areas between pits as convex membrane casts of the underlying particles, with the E-face pits occupying the points defining the base of the cast.

One possibility is that the pits are merely shadows behind the bulges. Alternative interpretations must take into account the composition of the material between the particles. Not much is known about this question. Low-irradiation electron microscopy of negatively stained liver gap junctions isolated with detergents shows a large variation in the amount of stain between connexons, with little stain in a triangular region at the threefold axis (Baker, et al., 1983). Peracchia and Girsch (1985) have presented preliminary evidence for filamentous bridges between particles of isolated liver gap junctions subjected to rotary shadowing and deep etching to the ES-surface after pulling the junctions apart with hypertonic sucrose. These

filaments (1.5 - 2.2 nm thick and ~1.5 nm long) were observed to join neighboring particles or to join end-to-end with other bridges. In stereo images the bridges were located at a level lower than the particle summits. Similar connecting structures have been detected in reconstructed images of isolated liver gap junctions studied by low dosage electron microscopy (Wrigley, et al., 1984).

The relationship of these as yet incompletely defined structures is speculative. The present data is confined to the location of the pits. The nature of the pits remains to be conclusively established.

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LEGENDS

Figure 1. Diagram illustrating the four-step sequence for aligning and mounting stereo images of complementary replicas to form an array of three photographs containing two stereo images as in Figures 5, 7,9, and 10. For details see text (technique 3).

Figure 2 Artist's drawing depicting a model of a gap junction that has been split into two complementary replicas (A and B) in the process of freeze-fracturing the two cells (C_1 and C_2) whose plasma membranes come together to form the junction. The cytoplasm of cell C_1 may be imagined to lie behind the upper part of the split junction (A); the cytoplasm of cell C_2 lies below the lower part of the junction (B). The lower part of the model shows predominantly the particulate P-face, the upper part the corresponding pitted E-face. For simplicity, the central depressions (which are not always seen on tops of the replicated particles, but are thought to be the central channels) are not indicated. The intercellular gap is shown in a cross-fracture of the junction on the right. Since the fracture plane was never seen to follow the gap, the gap is not shown where the junction is fractured <u>en face</u>. The gap is probably contained with the layer L, while the fracture plane steps from one side of the layer (L_1) to the other side (L_2). The presence of a common layer (L) separating the two sets of particles (P_1 and P_2) from the two cells (C_1 and C_2) becomes evident by three-dimensional visualization of the complementary replicas using stereo imaging as in Figures 4 and 6. The E-face always appears pitted after unidirectional shadowing, whether (as L_1) it belongs to cell 1 or (as L_2) it belongs to cell 2. The diagram illustrates a patch of the pitted layer L_1 which has become detached during fracture from the upper half (A) and remains

with the particulate surface (P_2), thereby leaving the central opening (window) in the pitted area of the upper half (A). Through this window a set of particles (P_1 of cell C_1) can be seen by examining the E-face of replica A. The detached patch overlies the particles (P_2 of cell C_2) that show up as the particulate P-face on replica B. The particles P_1 originate in the membrane of cell C_1, and the particles P_2 originate in the membrane of cell C_2; i.e., during freeze-fracture, the membranes separate so that P_1 travels with C_1 and P_2 travels with C_2. By contrast, during freeze-fracture the membranes separate so that the E-faces are exchanged between the cells: Viewed from above, the patch of pitted E-face labeled L_1 (which forms the top of a composite layer into which the particle of replica B can be seen to bulge) originated from the membrane of the upper cell (C_1). The pitted surface L_1 is the external leaflet (E-face) of the membrane of cell C_1. The area (L_2), which remains with the upper cell (C_1) after the "window" (L_1) has been removed, originates from the E-face of the membrane of the lower cell, C_2. See text for additional discussion.

Figure 3. Electron micrograph of a complementary replica of a freeze-fractured gap junction in a sheep cardiac Purkinje fiber. The pictures were mounted to display the symmetry of details of the replicas. The left panel is made up predominantly of the particulate P-face of the membrane; the right panel shows the pitted complementary E-face. Numerous caveolae present in the upper part of the micrographs also display symmetrical features. The dark bottom of the right panel is an artifact of fracturing the right replica. This is a non-stereo image. This and all subsequent electron micrographs have been double-printed so that shadows appear black. X 50,000

Figure 4. A set of two stereo pairs of the gap junctions shown in Figure 3. This set, as well as those in Figures 6 and 8, should be looked at with a stereo viewer to bring out the layered structure of the junction and to see how the junction splits to produce complementary replicas. The upper stereo pair shows mainly the particulate P-face of the membrane; the lower stereo pair shows mainly the E-face. The large, branched, particle-free area in the upper stereo pair is a patch of the E-face (layer L in Figure 2) that covers the particles underneath it. A similarly shaped opening ("window") through which P-face particles are visible in the E-face can be seen in the complementary replica (the stereo pair in the lower panel). Such stereo views show that these particles lie in a different plane than that of the P-face particles in the upper panel. X 50,000

Figure 5. The complementary gap junctional replicas shown in Figures 3 and 4, displayed as a sequence of three photographs containing two stereo images arrayed as in step 4 of Figure 1. This figure and Figures 7, 9, and 10 should be viewed with a stereo viewer in the manner explained in technique 3 in Methods and shown in Figure 1 in order to demonstrate that superimposition causes the E-face pits to fall in between the complementary P-face particles. Note that the particle pattern on the P-face is traceable on the E-face; for example, the small, particle-free oval area is also detectable under the E-face. Alternate stereo viewing of the right and left stereo pairs shows that, in favorable areas as on the right, the branched pattern of spaces between E-face pits is exactly filled with particles. The patterns of particle clusters can also be identified on the pitted areas. X 50,000

Figure 6. A set of two stereo images of complementary faces of a gap junction

which, on stereo viewing, displays marked curvature of its surface. A slight difference in slope produces a difference in shadowing angle that causes the pits to disappear in the upper region of the gap junction, whereas they are well visualized in the lower part of the patch of membrane. The clear outlines of the shapes of the patches of the E-face portions which appear on both complementary replicas facilitate identification of complementary areas on P- and E-faces. The most convenient areas for the purposes of such identification are those so small that they contain few particles or pits; such areas are suitable for matching at high magnification. The identifying landmarks are the structural details surrounding the areas to be matched, e.g., the edges of fractured membranes or the structures in the cytoplasms. X 100,000

Figure 7. Sequence of three panels for stereo viewing of complementary replicas mounted to be viewed as explained in Figure 1. At this higher magnification the location of pits in the spaces between particles is well demonstrated. The middle picture becomes filled with the particles from both faces, while at the same time it contains all the pits from both the left and right pictures. X 100,000

Figure 8. Two stereo pairs of complementary replicas of a gap junction mounted so as to display symmetry of details on both surfaces. This junction split in such a way that only the pitted E-face appears on one replica, and only P-face particles appear on the other replica. Stereo viewing clearly shows a second layer of particles under the E-face of the lower stereo pair. This second layer of particles manifests its presence by bulges in the area between pits. Arrows point to the extra-junctional landmark, a caveolar neck, which is also present in Figures 9 to 13. X 50,000

Figure 9. Triple array of the gap junction shown in Figures 8 - 11, mounted for stereo viewing as in Figure 1. In the central area of the gap junction, where the alignment is good, it is evident that the pits fall into the spaces between particles, not on them. X 50,000

Figure 10. A higher magnification of the array shown in Figure 9. X 100,000

Figure 11. A greatly magnified portion of the gap junction shown in Figures 8 - 10. This is not a stereo set, although the two complementary faces (right and left panels) were combined to produce the central panel. Some of the particles on the particulate surface (right) are missing, while others are obscured by heavy shadowing. The array of pits on the pitted surface (left) is more regular than the particle pattern (right). The middle panel shows how well the features of the right and left panels can be matched. These images should be compared with the tracings and "maps" of Figures 12 and 13. Next to the left margin of the gap junction, a fragment of the caveola (indicated by an arrow in Figure 8) marks the level of an unusual triangular pattern that can be used to compare the locations of the corresponding particles and pits in all three panels of Figure 11. This comparison of locations shows that the pits fall between the particles. Some of the particles are missing. X 400,000

Figure 12. High contrast enlarged reproductions made with a copier and enlarger of the right and left panels in Figure 11. The right panel shows a "map" made by numbering the particles; the left panel shows the areas between pits corresponding to the numbered particles on the right (see text). The outline of a caveola (shown by arrow in Figure 8) is evident at the left margin of both panels.

Figure 13. Diagrams showing the steps in the procedure for matching the structural features of the complementary gap junctional E-face and P-face shown in Figures 8 - 12. See text for description (technique 5).

Table 1

Measurements on Complementary Replicas of Gap Junctions

Gap Junction #	Mag. ($\times 10^{-3}$)	Fracture Face Area Examined (μm^2)	Number of Particles or Pits in the area	Number/μm^2	NND*	$\frac{\text{Number of pits}/\mu m^2}{\text{Number of particles}/\mu m^2}$
1 (fig. 7)						
particles	750	.045	571	12.8×10^3	6.280	1.34
pits	750	.045	763	17.1×10^3	6.024	
2 (fig. 7)						
particles	510	.021	177	8.57×10^3	8.017	1.37
pits	510	.021	242	11.8×10^3	6.20	
3 (fig. 8)						
particles	912	.033	344	10.3×10^3	6.541	1.51
pits	912	.033	521	15.6×10^3	6.133	
4 (not shown)						
particles	760	.043	105	9.70×10^3	7.025	1.50
pits	760	.044	158	14.4×10^3	5.611	

* Mean nearest neighbor distance (Page, et al., 1983)