Patterned Growth of Neonatal Rat Heart Cells in Culture
Morphological and Electrophysiological Characterization

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A culture method was developed that permits patterning of the growth of ventricular myocytes of neonatal rats. Regions were created on the culture substrate that either prevented (photore sist coat) or supported (glass surface) attachment of cells. In this way the geometry of interconnecting growth channels could be specified. Single-layered myocyte strands of variable length and with widths of as little as 65 μm (three to four cells wide) were obtained. The shape and orientation of the individual myocytes were a function of growth-channel width: the narrower the channel, the more elongated the cells and the more likely was the long axis to be oriented along the channel axis. In channels with widths of 100 μm or less, cells were aligned longitudinally and cross-striated as in vivo. A high degree of morphological cell differentiation required the presence of contractile activity. Maximal diastolic potential (−71 mV), action potential amplitude (93 mV), and maximal upstroke velocity (140 V/sec) did not change with increasing culture age. Mean longitudinal conduction velocity was 0.39 m/sec. No electrophysiological or morphological evidence of photore sist toxicity was seen, and the data indicate a high degree of cell differentiation in the patterned cell cultures. The method thus is suitable for the study of the relation between impulse propagation and structure at a cellular level in artificial networks of predefined shape. (Circulation Research 1991;68:114–130)

The structure of the excitable tissue influences impulse conduction in the heart. The longitudinal shape of the ventricular myocytes, their interconnections, and their assembly into strands determine the anisotropic behavior of passive electrical properties, action potential upstroke, and propagation. Thus, intercellular resistance and action potential upstroke depend on the direction of subthreshold or depolarizing current flow, and conduction velocity along the longitudinal axis of ventricular fibers is two to three times higher than along the transverse axis. Despite the considerable heterogeneity of cells at a microscopic level, decay of subthreshold currents and action potential propagation in the longitudinal direction are well described by a continuous linear cable model in the case of normal ventricular tissue and Purkinje fibers. Complex extracellular wave fronts, indicating discontinuities of propagation, have been described only for transverse propagation in ventricular strands or conduction in the intermingled atrial trabecula.

Computer modeling of impulse conduction in cardiac tissue has shown that evidence of discontinuous propagation, such as discontinuities of action potential upstroke, can be produced by creating localized depression of excitability or decreased cell-to-cell coupling. Conduction delays at such sites may lead to unidirectional conduction block or to electrotonic reflection of an action potential. Both have been invoked as main determinants of reentry and tachycardias observed in vivo. The experimental assessment of circus movement reentry in many forms of atrial tachycardia, acute myocardial ischemia, or myocardial infarction is straightforward, because the diameters of the circulating wave fronts are relatively large (several millimeters). Moreover, in some of these experimental settings, it is possible to reduce the excitable tissue to a two-dimensional sheet that eliminates the problem of three-dimensional mapping of propagation.

One of the experimental limitations to date has been the spatial resolution of the mapping techniques. The spacing between recording electrodes exceeds the length of a single cell severalfold, and it is difficult to correlate the microscopic tissue archi-

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tecture with the multiple recording sites. These limitations may be especially important in those forms of conduction disturbances that are confined to small tissue regions (so-called focal-reentry or microreentry) such as the specialized ventricular conducting system or the atrioventricular node.

In this work, we describe a method developed with the ultimate aim of assessing the relation between conduction of the impulse and cytoarchitecture in cultured cell patterns at a microscopic level. A similar experimental approach has previously been used in heart by Lieberman and coworkers to culture cablelike myocardial strands. Our technique permits patterning of the growth of myocytes in culture in linear channels and complex two-dimensional networks. The exact pattern of the culture substrate can be predefined and can be made comparable to a computer-simulated network. The morphological and electrical characteristics of the cells forming the growth patterns indicate a high degree of morphological and functional differentiation.

Materials and Methods

Lithographic Fabrication of Patterned Coverslips

The main steps in the preparation of patterned cell coverslips are shown in Figure 1.

Conventional glass coverslips were patterned by photolithographic techniques with a material to prevent cell adhesion. Preliminary experiments were undertaken to evaluate a photoresist that 1) adhered to glass under conditions of water immersion, 2) prevented the adhesion of dissociated cells, and 3) had no deleterious effect on the gross cellular morphology. Of 13 tested photoresists, KTFR (Kodalith, Lausanne, Switzerland) best met these specifications and was used for the development of a standard coating procedure:

Photoresist coating. Circular glass coverslips (22-mm diameter, Haska, Bern, Switzerland) were carefully cleaned to remove dirt and organic matter (rubbing the surface with acetone, subsequent washing in H₂SO₄, and final neutralization with NaOH).

After drying at 200°C for 60 minutes, they were stored in a desiccator and subjected to a second identical dehydration process immediately before the application of the photoresist. The photoresist was handled under low-intensity yellow light illumination (fluorescent tubes TLD 18W-16, Philips, Zurich, Switzerland) that did not interfere with its spectral sensitivity. A droplet of resist was applied to the center of a glass coverslip that was horizontally mounted in a custom-made spinning device. Uniform coating of the glass coverslip was achieved in two steps: the coverslip first was spun for 5 seconds at 500 rpm to spread the photoresist over its entire surface, and then for 60 seconds at 3,000 rpm to distribute the photoresist uniformly and to reduce its thickness to 1 μm or less. Solvents were removed by heating the coated coverslips for 10 minutes at 90°C (prebaking). Subsequently, the coated coverslips were sealed in a light-tight container and placed in a desiccator until exposure.

Pattern design. Growth patterns were designed on a personal computer (Macintosh II, Apple Computers, Wallisellen, Switzerland) using commercially available computer-aided design programs. The patterns were printed on a laser printer (Laserwriter IINT, Apple Computers) and photographically reduced to match the size of the glass coverslips (Kodalith Ortho Film 6556 type 3, Kodak). The film negatives were used directly for the exposure process.

Exposure and development. Photoresist-coated coverslips were contact exposed to the desired pattern in a UV light copier (AV-Berna, Berna, Bern, Switzerland). The illumination spectrum of this copier box covered the photosensitive range of the resist (maximal sensitivity at 400 nm). After exposure, coverslips were mounted in a rack, developed in a bath for 1 minute, rinsed with distilled water, and individually dried with pressured air. This procedure resulted in a pattern consisting of previously UV light-stabilized photoresist regions and regions where the glass surfaces of the coverslips were uncovered. The subse-
frequent heating at 120°C for 10 minutes (postbaking) increased the durability of the processed photoresist. Patterned coverslips were kept in a desiccator until further use and were sterilized at 120°C for 2 hours before the establishment of cultures.

**Experimental test pattern.** Figure 2 depicts the design of the test pattern used for morphological and electrophysiological measurements. Parallel straight channels having widths of 65 μm (W65), 100 μm (W100), or 150 μm (W150) extended fingerlike from a broad peripheral annulus into the photoresist-coated center of the coverslip. The ratio of coated to uncoated areas was approximately 1:3.

**Preparation of Cultures**

**Dissociation.** Dissociated heart cells were obtained using established techniques. In brief, hearts from eight neonatal Wistar rats (2–3 days old) were removed and finely minced. The pieces were immersed in a dissociation solution (Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution) (Gibco, Basel, Switzerland) containing 0.1% trypsin (Boehringer, Rotkreuz, Switzerland), 60 μg/ml pancreatin²⁰ (4×N.F. grade, Sigma Chemie GmbH, Deisenhofen, FRG), 20 units/ml penicillin (Fakola AG, Basel, Switzerland), and 20 μg/ml streptomycin (Fakola), and repeatedly stirred for 15 minutes at 35°C. Five to six incubation periods were required to dissociate the tissue completely. The solutions obtained after all except the first dissociation steps were decanted into tubes and sedimented by centrifugation. The resulting cell pellets were resuspended in medium M199 (Gibco) having an ionic composition of (mM) NaCl 137, KCl 5.4, CaCl₂ 1.3, MgSO₄ 0.8, NaHCO₃ 4.2, KH₂PO₄ 0.5, Na₂HPO₄ 0.3, and containing 20 units/ml penicillin, 20 μg/ml streptomycin, 2 μg/ml vitamin B₁₂ (Sigma), and 10% neonatal calf serum (Boehringer). This medium will be referred to as HSM (high serum medium), whereas the same medium containing only 5% serum will be denoted as NSM (normal serum medium).

**Preplating.** The fibroblast content of the cell suspension was reduced using a differential attachment method.²¹ The cell suspension was transferred to a 150-cm² culture flask (Sterilin, Amimed AG, Muttenz, Switzerland) and was kept for 2 hours in an incubator (Jouan EG 110, Lightning Instrumentation, Lausanne, Switzerland) in an atmosphere of water-saturated ambient air containing 1.7% CO₂. After this incubation (preferential fibroblast adhesion to culture flask), the myocytes remaining in suspension were counted with a hemocytometer and diluted to achieve 3×10⁵ cells/ml. Simultaneously with the differential attachment procedure, patterned coverslips and control coverslips (no photoresist coating) were transferred to multiwell plates (Linbro 12-well plates, Flow Laboratories, Allschwil, Switzerland) and preincubated with 1 ml HSM for 2 hours; this preincubation increased the percentage of adhering cells.

**Final plating.** After the preincubation medium was withdrawn from the multiwell plates, 2 ml of the enriched myocyte suspension was placed into each well, and the plates were transferred for 20–24 hours to the incubator. After this, the plates were gently shaken to bring sedimented but unattached cells into solution, and the medium was exchanged for NSM. Thereafter, medium exchanges with NSM were performed every other day. Up to 20 patterned coverslips were obtained with each individual culture.

**Electrophysiology**

**Measurements.** Measurements were carried out in a temperature-controlled experimental chamber mounted on an inverted microscope (IM 35, Zeiss, Zurich, Switzerland). Phase-contrast illumination was used for electrophysiological and morphological measurements. Specimens were superfused with NMS at 37±0.1°C at a rate of approximately 1 ml/min. Microelectrodes (GC 120F-10, Clark Electromedical Instruments, Reading, UK) were fabricated on a horizontal puller (Mecanex BB-CH, Mecanex, Nyon, Switzerland), filled with 3 M KCl, and mounted on a micromanipulator (MR MOT, Zeiss). The signals from the electrode amplifier (model 750, W-P Instruments Inc., New Haven, Conn.) were displayed on a digital storage oscilloscope and recorded on tape (VR-10, Instrutech Corp., Mineola, N.Y.) for later off-line analysis.

For the measurement of upstroke velocity and action potential amplitude, care was taken not to overcompensate the capacity of the recording electrode. A test setup using the method of Nastuk and Hodgkin indicated that, at superfusate levels of 3 mm or less, electrodes with resistances of 40 MΩ or less could not be overcompensated with the headstage and amplifier combination used (maximal error ≤5%). Electrodes ranging near this resistance limit were selected because fine tips were a prerequisite.
for successful impalements (mean resistance±SEM for all electrodes, 42.2±1.4 MΩ).

**Experimental protocol and data analysis.** A total of seven cultures was studied. In each culture, electrophysiological measurements were carried out on days 2, 4, 6, 8, 11, 14, and 17. On each of these days, up to three successful impalements were obtained in each growth-channel type (W65, W100, W150) and in the control monolayer. After the measurements, the coverslips were discarded because of potential microbital contamination. Only tracings from stable impalements lasting for at least 1 minute (mean, 3.0±0.2 minutes) were analyzed. Signals replayed from tape were differentiated by a custom-made device (linear up to 1,000 V/sec) and fed to a transient recorder (SMR-2, Scientific Instruments, Basel, Switzerland) for analysis. Action potential durations were assessed as time intervals between the rapid portion of the action potential upstroke and repolarization to 20% (APD<sub>20</sub>) and 80% (APD<sub>80</sub>) of the action potential amplitude.

**Morphology**

**Image acquisition and experimental protocol.** Pictures from cultures (magnifications ×63, ×160, or ×400) were recorded using a video camera (WV-CD52, Panasonic, Wettingen, Switzerland) attached to the microscope (phase-contrast illumination). Before still photographs were taken, growth channels of patterned cultures were aligned to a horizontal grid in the microscope to enable calculations of cell orientation (see below). The term “horizontal” in the subsequent text refers to lines parallel to this grid. The video signal was displayed on a monitor (WV-5350, Panasonic) and digitized by a frame grabber card (QuickCapture, Data Translation, Thalwil, Switzerland) installed in a personal computer (Macintosh II). Pictures were stored on a removable hard disk (Spark44, Spark International, Glenview, Ill.), and hard copies were obtained from a videographic printer (UP-811, Sony, Baden, Switzerland). Three pictures were obtained from each growth-channel type and from the control monolayer after completion of the electrophysiological measurements. The experimental protocol is therefore identical with that of the electrophysiological studies (see above).

**Cell type identification.** Myocytes (MCs) were identified based on well-known morphological criteria. They displayed a dense granular cytoplasm with often prominent cross striations and had a compact round nucleus that usually contained only one nucleolus. With increasing age, MCs tended to become binucleated, a feature also observed in vivo. In contrast, fibroblasts had an irregular shape with multiple filopodia, and their cytoplasm was phase-lucent and unstructured. If cytoplasmic granulations were present, they were arranged around a single oval-shaped nucleus often containing two nucleoli. Epithelioid cells resembled fibroblasts and typically formed confluent islets consisting of similarly sized polygonal cells. Epithelioid cells and fibroblasts were assigned to the group of nonmyocyte cells (NMCs).

**Computer-assisted image analysis.** The contours of individual MCs were outlined on the computer screen using a graphics tablet (Bit Pad Plus Mac, Summagraphics, Zurich, Switzerland). Based on this outline and the appropriate scaling factors, the image analysis software (OPTILAB, Grafeik, Orsay, France) computed cell-specific parameters in appropriate units as defined on Figure 3. The cell area (in square micrometers) corresponded to the area enclosed by the cell contour, and the cell length (in micrometers) was equal to the longest straight line within a cell contour. The cell width (micrometers) was equaled to the minor axis of the cell equivalent ellipse, that is, an ellipse defined by the cell area and the cell length. The orientation of the cell was calculated as deviation of the cell long axis from horizontal (=channel axis, see above) in degrees (=angle α).

**Assessment of nonmyocyte cell contamination.** The evolution of the NMC contamination in the different channels and in controls was assessed in an additional culture on each of days 2, 4, 6, 8, 11, 14, and 17 based on the cell type identification criteria described above. Each growth-channel type was scanned at highest magnification (×400) until a total count (MCs plus NMCs) of 50 was reached. This was repeated at least 10 times, resulting in 500 or more counted cells per day and channel type. The same counting scheme was adopted for control monolayers up to the sixth day; that is, 50 contiguous cells were counted in at least 10 arbitrarily selected microscopic fields. After the sixth day, all cells in a series of arbitrarily selected microscopic fields were counted up to a cell count of 500 or more. This was necessary to avoid observer bias, because NMCs and MCs were progressively distributed nonhomogeneously after the sixth day in control monolayers.

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Parameter Definition</th>
<th>Units</th>
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<tbody>
<tr>
<td>A</td>
<td>cell area</td>
<td>grey shaded area</td>
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<tr>
<td>b</td>
<td>cell length</td>
<td>longest straight line within cell</td>
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<td>c</td>
<td>cell width</td>
<td>length of minor axis of cell equivalent ellipse (eq-E): ellipse area = cell area; ellipse major axis = cell length</td>
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<tr>
<td>a</td>
<td>cell orientation</td>
<td>deviation of the direction of the cell length from horizontal</td>
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*Figure 3. Definition of morphometric cell parameters (for further explanation see text).*
Statistical Analysis

Results are expressed as mean±SEM. Differences between means were tested by analysis of variance followed by Scheffe’s multiple comparison test. Trends depending on increasing culture age were tested by regression analysis. The level of significance was set to p<0.01.27

Results

Structure of Blank Test Pattern

The scanning electron micrograph in Figure 4A depicts a detail of the photoresist pattern on a coverslip. It shows the region of connection of a presumptive growth channel to the peripheral ring. The picture illustrates that the photoresist was gradually etched down to the glass at transition sites resulting in thin and narrow (<7 μm) zones of photoresist flanking the glass surface of the growth channel. This produced the light-microscopic illusion of the MCs slightly retracting from the photoresist, because they did not spread onto this phase-lucent narrow zone.

Morphology of Patterned Myocytes

A partial overview of directed growth obtained with the test pattern is shown in Figure 4B. The attachment of the evenly sedimented cells and their subsequent growth was confined to the exposed glass surfaces. This resulted in cell strands (or two-dimensional networks) in the patterned central region that were connected to a peripheral cell monolayer. Only a few cells adhered to the photoresist-coated areas. The light-microscopic appearance of a control monolayer culture is illustrated in Figure 4C. Individual MCs formed a network and showed no preferential orientation. The cells were polymorphic, and some of them displayed cross-striations. In Figures 4D–4F, representative examples of the three different channels (W65, W100, W150) are shown in detail. The cells filled the whole width of the channels, and individual, mostly cross-striated MCs could be defined easily. The overall shape of the MCs was ellipsoid, and their degree of longitudinal alignment was dependent on the channel width. In narrow channels (W65, W100), all MCs were aligned along the axis of the channels. In wider channels (W150), only cells at the periphery were strictly longitudinally aligned, whereas the more central cells deviated to various degrees from the long axis of the channels. There was no significant overlap of individual MCs as judged from scanning electron micrographs (data not shown) and light microscopic examinations.

The evolution of the MC cell area for the three different strand widths and for controls is shown in Figure 5A. By day 17, the cell area of all growth types had significantly increased by approximately 45% when compared with initial values on day 2. With the exception of three isolated cases (day 8, W150; day 14, W100 and W150), the area of the control cells was always significantly larger than that of the patterned cells. Among the patterned cells, a consistent direct relation between the channel width and the mean cell area could be observed.

The values for MC length showed a significant tendency to increase with time in culture for all three channel widths as well as for controls (Figure 5B). Cells elongated significantly from initial values of approximately 50 μm to values of approximately 70 μm on day 17 (mean increase, 40%). Neither initial length nor the elongation trend was dependent on the pattern types, resulting in significant differences in cell lengths in only two cases (day 4, control versus W100; day 17, W100 versus W150).

In contrast to MC area and length, MC width showed no tendency to change with increasing culture age (Figure 5C). Values averaged over the whole observation period ranged from 11 μm (W65) to 16 μm (control). Control cells were always significantly wider than patterned cells in all but one case (day 14, control versus W150). As in the case of the cell area, there existed a consistent direct relation between the channel width and cell width throughout the experimental period. This was substantiated by the finding that values of W65 were always significantly smaller than those of W150.

As expected, MCs in control monolayers had no preferential orientation (mean angle of deviation, 45°; Figure 6). In contrast to control, patterned MCs consistently showed smaller angles; that is, they were oriented to a substantial degree along the long axis of the channels. The amount of deviation found in the three different pattern types decreased significantly with increasing culture age. This tendency was especially prominent for W100, which showed a decrease of angle α during the whole observation period from 25° to 10°. On all days, the degree of deviation was directly related to the channel width. This relation is corroborated by the constantly significant differences between W65 and W150 (except day 14).

Proliferation of Nonmyocyte Cells

Qualitative examination of all cultures revealed a low degree of contamination of the growth channels with NMCs. When present, fibroblasts were exclusively observed flanking the MC strands (arrow in Figure 4D), whereas epithelioid cells on very rare occasions interrupted MC strands by forming typical multicellular islets in the growth channels. The MC strands on both sides of such groups of interposed epithelioid cells usually beat synchronously. The dependence of the percentage of NMCs on culture age is shown in Figure 7. In controls, NMC contamination was less than 10% until the sixth day, and the different cells were homogeneously distributed. Thereafter, the MCs showed a progressive tendency to cluster, and concomitantly, dividing NMCs began to form islets of up to 30 cells. Such a tendency could not be observed in the patterned MC strands. NMC contamination in control cultures after the sixth day was always significantly higher than that in patterned MC strands and finally accounted for 40% of the
whole cell population. In contrast to controls, NMC contamination in the different channels was low and varied between 2% and 7% with the exception of the culture dishes measured on day 14 (>10%). There existed no significant differences among the different channel types.

In addition, we could not confirm the observation of other authors\(^{28,29}\) that fibroblasts rapidly overgrew the MC layer. In our cultures there was neither light-microscopic nor scanning electron-microscopic evidence for a stratification of the cultures by NMCs overgrowing the MCs, not even after extended culture periods.

**Influence of Contractile Activity on Myocyte Morphology**

An interesting observation was made in those cell strands that were not electrically coupled to the ring impulse generator (=circumferential MC monolayer). This finding is illustrated in Figure 8 by the micrographs of two W65 channels of the same culture dish. The morphology normally encountered in these channels is depicted in Figure 8A and is characterized by well-differentiated, longitudinally aligned cells having an ellipsoid shape. This strand was visibly connected to the impulse generator and was beating at a regular rate. On rare occasions, however, cells with an entirely different morphological aspect were observed, as shown in Figure 8B. These cells displayed a low degree of morphological differentiation; that is, they were smaller, irregular in shape, displayed no cross-striations, and were not longitudinally aligned. Such cell strands were always visibly separated from the impulse generator by a cell-free gap and were quiescent. This suggests a close correlation between contractile activity and cell differentiation.

**Complex Growth Patterns**

Networks composed of rectangular subunits were designed to test the feasibility of this method of constructing complex growth patterns. Examples for such patterns are shown on Figure 9. The overview in Figure 9A illustrates that a largely uniform patterning of MCs was achieved over extended areas of the culture substrate. The enlarged view of the same culture dish in Figure 9B shows that these small photoresist rectangles of the dimensions of a few cells were capable of patterning the arrangement of MCs precisely. The white spots on the photoresist shown in Figure 9A correspond to cellular debris in most cases. Most of this debris was removed during repeated medium replacements. Figure 9C depicts a similar pattern as shown in Figure 9B, except that the side lengths of the individual rectangles were enlarged from 140×330 to 400×640 μm, while the growth-channel widths remained the same. This change resulted in more longitudinally aligned MCs.

**Electrophysiology**

Membrane potential parameters were measured in spontaneously beating preparations (140±5 beats/min).

**Maximal diastolic potentials.** The maximal diastolic potentials ranged from -67 to -76 mV (mean, -71.0 mV; Figure 10, lower panel) and showed no tendency to change with culture age. Patterned cells did not differ significantly from controls and were not significantly different among themselves. Small afterhyperpolarizations of a few millivolts occasionally were observed in rapidly beating preparations. The majority of cells showed no phase 4 depolarization.

**Action potential amplitudes.** Action potential amplitudes varied between 83 and 98 mV (mean, 92.9 mV; Figure 10, upper panel) and showed no significant tendency to change with increasing culture age. Values of patterned cells did not differ from control values with one exception (day 8, W100 versus control). No significant differences were found among the patterned MCs.

**Upstroke velocities.** Values for upstroke velocities ranged from 65 to 228 V/sec (mean, 140.5 V/sec; Figure 11). Upstroke velocities of W65 and W100 showed a significant tendency to decrease with culture age, whereas those for W150 and controls remained stable throughout the experimental period. Upstroke velocities of the patterned cells differed significantly on four isolated occasions from control (day 8, W150 versus control; day 14, all channel types versus control). The values among the different channels never differed significantly.

**Action potential durations.** APD\(_{20}\) and APD\(_{90}\) of all cells measured decreased significantly with culture age as shown in Figure 12. After an initial phase, during which the action potential durations of the patterned cells were significantly longer than those of the controls, they became, with the exception of day 14, similar to those of the controls, indicating no major change in the action potential shape under the various conditions.

**Conduction velocity.** In one culture, conduction velocities in different channel types were assessed on several days using a local extracellular stimulating electrode and a remote intracellular electrode. The strands were stimulated at overdrive frequencies by application of square constant-current pulses of 2-msec duration at double threshold intensity. The intracellular electrode was placed as far as possible from the stimulating electrode and then was moved toward the latter in steps less than or equal to 1 mm. Conduction velocities were calculated from the corresponding differences in activation times. The mean value obtained from all 39 measurements was 0.39±0.19 m/sec (mean±SD; range, 0.09–1.03 m/sec).

**Discussion**

**Fabrication of Patterned Culture Substrates**

The ability of dissociated cells to adhere to specific surface structures has been used by several investigators to pattern the growth of cells in cultures derived from different tissues: neurons,\(^{30–32}\) MCs,\(^{15–18,30,33,34}\) and fibroblasts.\(^{35,36}\) Patterning of heart cells in culture to produce “unidimensional cablelike” cell strands was
FIGURE 4. Photomicrographs of patterned growth. Panel A: Scanning electron micrograph of the connection of a 65-µm-wide growth channel to the peripheral glass ring before cell plating (calibration bar=40 µm). Panel B: Partial overview of patterned cell culture (8 days old) showing the peripheral cell monolayer ring and the emerging cell strands between interposed photore sist-coated areas (calibration bar=400 µm). Panel C: Tightly packed polymorphic myocytes of 8-day-old control monolayer culture (calibration bar=40 µm). Panels D, E, and F: Myocyte strands (11–15 days old) growing in 65-µm (panel D), 100-µm (panel E), and 150-µm-wide (panel F) channels. Note increasingly parallel orientation of cells with decreasing channel width (same calibration as panel C). Arrow in panel D denotes fibroblast.
Figure 5. Column graphs showing morphological parameters as a function of culture age and growth pattern: control monolayer (black columns), 65-μm-wide channels (dark gray shaded columns), 100-μm-wide channels (light gray shaded columns), and 150-μm-wide channels (white columns). Numbers above data columns indicate number of analyzed cells. Significant tendencies of the parameters to change with culture age are marked by an asterisk above the data columns of the growth patterns concerned on day 17. Square matrices below column graphs show results of statistical comparisons among the four different growth patterns for each age (significances are denoted by X, lack of significance by O). Panel A: Cell area (mean±SEM) as a function of culture age. There exists a consistent direct relation between channel width and mean cell area, and the area shows a significant tendency to increase with culture age for all growth patterns. Panel B: Cell length (mean±SEM) as a function of culture age. There is a significant tendency of the length to increase with culture age for all growth patterns. For a given age, no difference in cell length among the four patterns is found. Panel C: Cell width (mean±SEM) as a function of culture age. There exists a consistent direct relation between channel width and mean cell width for all given ages. Cell width does not change significantly with increasing culture age in any of the four growth patterns.
first performed by Lieberman and coworkers,\textsuperscript{15–18} who grew dissociated cells in grooves cut in agar\textsuperscript{15–17} or used nylon filaments as substrates.\textsuperscript{18} The aim of the present work was to develop a method for the production of two-dimensional, reproducible growth patterns of MCs in culture. This was achieved by using a glass coverslip differentially coated with photoresist to prevent cell adhesion at predefined sites. Photolithographic coating and processing of culture substrates to control the outgrowth of dissociated neurons in culture was first reported by Kleinfeld et al.\textsuperscript{32} In their method, hydrophilic and hydrophobic ligands were chemically bound to a glass surface at sites predefined by a photographic mask. This method involves several relatively complex steps of fabrication. It has the advantage that the chemical composition of both the cell-adhering and the cell-rejecting substrate layers is well defined. The method developed in the present study involved a single-step photolithographic coating procedure with a hydrophobic polymer of a chemical composition that was not precisely specified by the manufacturer. Therefore, possible toxic effects on morphology and electrophysiology of the cells adhering to the glass bottom of the growth channels had to be excluded. The comparison of the results obtained with both methods demonstrates that in either case, two-dimensional cellular

\begin{figure}
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\caption{Column graphs showing deviation of long cell axis from horizontal (=angle $\alpha$; mean $\pm$ SEM) as a function of culture age and growth pattern: control monolayer (black columns), 65-\mu m-wide channels (dark gray shaded columns), 100-\mu m-wide channels (light gray shaded columns), and 150-\mu m-wide channels (white columns). Numbers above data columns indicate number of analyzed cells. Significant tendencies of the parameters to change with culture age are marked by an asterisk above the data columns of the growth patterns concerned on day 17. Square matrices below column graphs show results of statistical comparisons among the four different growth patterns for each age (significances are denoted by X, lack of significance by O). At a given age, cell deviation is directly related to width of the growth channels. With increasing culture age, cells in all channel types exhibit a significant trend toward an increased longitudinal orientation.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure7}
\caption{Column graphs showing nonmyocyte cell (NMC) content (mean $\pm$ SEM) as a function of culture age and growth pattern: control monolayer (black columns), 65-\mu m-wide channels (dark gray shaded columns), 100-\mu m-wide channels (light gray shaded columns), and 150-\mu m-wide channels (white columns). Numbers above data columns indicate number of analyzed microscopic fields. Significant tendencies of the parameters to change with culture age are marked by an asterisk above the data columns of the growth patterns concerned on day 17. Square matrices below column graphs show results of statistical comparisons among the four different growth patterns for each age (significances are denoted by X, lack of significance by O). After the sixth day, the number of NMCs in control monolayer cultures increases rapidly but remains low in growth channels. See text for further explanation.}
\end{figure}
patterns of any shape (single channels, bifurcations, complex networks) can be obtained with a reproducible accuracy of a few micrometers.

**Morphology of Cells in Growth Channels**

The basic growth pattern used for the morphological and electrophysiological analysis was composed of two main elements: 1) a relatively large annulus-shaped monolayer, and 2) a central area containing the test channels of various widths. The circumferential spontaneously active monolayer served two purposes. First, the monolayer covered approximately 75% of the culture substrate and therefore comprised enough cells to effectively condition the culture medium. This conditioning was necessary because it had been shown that a cell culture must comprise a minimal number of cells to survive, an observation attributed to the medium-conditioning effect of a critical cell mass.24,37,38 Second, the spontaneously active ring monolayers delivered impulses that excited the normally quiescent cells in the growth channels. As shown in Figure 8, this was of critical importance for the morphological differentiation of the MCs: in those rare cases in which the cells in the growth channels were quiescent, that is, disconnected from the circumferential "impulse generator," the shapes of the cells even in the narrowest channels were polymorphic, they displayed no preferential direction, and their cytoplasms showed no cross-striations even after extended culture periods. This is in accordance with the observation that contractile activity favors the differentiation of myofilamentous material39,40 and the size of cultured cells.31 Additionally, it has been shown recently that contractile activity42 or repetitive passive stretch43 enhances the protein content of ventricular cells.

Continuous cell strands extended in most cases over the whole length of the channels (≤10 mm) and were, in the case of the smallest channels, composed...
**Figure 9.** Different aspects of complex growth patterns. Panel A: Overview of a two-dimensional network with cell adherence in 140-μm-wide growth channels and cell rejection on photoresist rectangles of 140×330 μm (day 3; calibration bar=800 μm). This overview illustrates that constant cell densities can be obtained over the whole surface of the patterned culture substrates. Most of the bright particles present on the photoresist coating correspond to cell debris (see also panel B). Panel B: Detail of panel A at higher magnification and with enhancement by phase contrast. The small photoresist rectangles precisely pattern the growth of the myocytes (day 10; calibration bar=200 μm). Occasionally, a nonmyocyte cell can be observed spanning a photoresist corner (arrows). Panel C: Picture of a different pattern: growth channel width is the same as in panel B but adhesion-rejecting rectangles are larger (400×640 μm). Compared with panel A, the wider spacing of the branching points results in an improved longitudinal alignment of the cells (day 15, calibration bar=200 μm).
of four to five cells aligned in parallel. In all three widths of growth channels and in the control monolayers, the area of the cells increased with culture age. This finding is in accordance with observations of other authors. It indicates that the increase in size of individual cells was independent of the growth pattern and excludes major toxic effects of the photoresist. Length and width of the patterned MCs compared closely with values found in vivo. Mean cell length in our cultures increased from 50 μm (day 2) to 69 μm (day 17). This was within the range described in vivo (22–44 μm, neonatal rat heart; 73–98 μm, adult rats). Values given for cell width in vivo (7–9 μm, neonatal rat heart; 15–18 μm adult rats) also were comparable to the mean values found in the growth channels (13 μm).

The morphology of the cells in the growth channels varied significantly among the different channels and differed from control monolayers. The main differences were in cell orientation and cell area. The degree of cell orientation was directly related to channel width, and a nearly parallel alignment along the axis of the channels was achieved in growth channels less than 100 μm. Regardless of the channel width, however, the cells in the immediate vicinity of the channel borders were always longitudinally aligned. This suggests that a lateral guidance of the MCs is necessary to achieve an in vivo–like arrangement of cells. An additional factor relevant for the longitudinal alignment was, as outlined above, the regular contractile activity of the cells in the channels during the whole culture period.

The cell area of patterned cells was always smaller than that of control cells, and it was directly related to channel width at any given culture age (area W65 < W100 < W150 < control). These differences were entirely attributable to concomitant differences in cell widths, because cell lengths were comparable among the four growth types. This suggests that lateral hindrance imposed by the growth channels was the major determinant of cell width and consequently of cell area. Such lateral hindrance, caused by neighboring cells, also is likely to determine the sizes of MCs in monolayer cultures. This suggestion derives from the finding that the MCs of our control monolayers were considerably smaller than those described by Simpson et al in sparsely seeded cultures (20% of our seeding density) but agreed well with values reported later by Simpson for MCs in cultures seeded at densities comparable to ours.

**Figure 10.** Column graphs showing action potential amplitudes (upper panel; mean±SEM) and maximal diastolic potentials (lower panel; mean±SEM) as a function of culture age and growth pattern: control monolayer (black columns), 65-μm-wide channels (dark gray shaded columns), 100-μm-wide channels (light gray shaded columns), and 150-μm-wide channels (white columns). Numbers above the data columns indicate number of analyzed cells. The small asterisk denotes the only value significantly different from control.

**Figure 11.** Column graphs showing maximal upstroke velocities of the action potentials (mean±SEM) as a function of culture age and growth pattern: control monolayer (black columns), 65-μm-wide channels (dark gray shaded columns), 100-μm-wide channels (light gray shaded columns), and 150-μm-wide channels (white columns). Numbers above data columns indicate number of analyzed cells. Small asterisks denote significant differences from control monolayer at a given age. Large asterisks above data columns on day 17 denote significant trends with culture age for a given growth pattern.
might ask in this context whether the reduction of cell width in the small growth channels was accompanied by an increase in cell depth, although a quantitative assessment of cell depth was not technically possible in the present experiments.

In cultured cell monolayers, contamination by NMCs represents a major problem for electrophysiological studies. Hyde et al.\textsuperscript{28} were the first to describe electrical coupling between fibroblasts and MCs, which caused alterations in the electrophysiological behavior of the latter. For the development of the present technique, it therefore was important to keep the contamination by NMCs as low as possible. This was achieved by preplating\textsuperscript{21} and by seeding cells at a high density.\textsuperscript{47} The combination of these two techniques yielded a high and stable percentage of MCs in the growth channels at any given culture age in the absence of antimitotics. Moreover, no overgrowth with NMCs\textsuperscript{28,29} was observed in the growth channels. In contrast to this stable composition found in growth channels, NMCs and MCs in control and circumferential monolayers became progressively nonhomogeneously distributed with increasing culture age. MCs began to form clusters interconnected by contractile cell strands, and the resulting MC-free regions became overgrown rapidly by proliferating NMCs. This resulted in a substantial increase of the percentage of NMCs after a few days in culture. A similar finding of NMCs rapidly occupying cell-free regions was made in the few cases of growth channels in which the cell strands broke apart. The disruption, which probably was induced by vigorous contractile activity, resulted in cell-free regions that became quickly and selectively overgrown by NMCs. These two observations suggest that the occurrence of cell-free regions was a prerequisite for NMCs to proliferate and is likely to explain the low NMC content of the growth channels where available space was always tightly filled with MCs and where the directing influence of the growth channels prevented a cellular rearrangement during the whole culture period. When fibroblasts were observed in growth channels, their growth was always confined to the channel borders; that is, they flanked the MC strands. On the few occasions in which MC strands were completely interrupted by interposed islets of NMCs, the strands continued to beat synchronously and thereby qualitatively confirmed electrical coupling between NMCs and MCs.\textsuperscript{28,48}

The growth patterns used for the determination of electrophysiological and morphological parameters differed from the linear cell strands produced by Lieberman et al.\textsuperscript{15} only in their thickness. The cell strands obtained were composed of only one cell layer and therefore allowed the identification of every single cell contributing to the whole structure. In addition, the method presented allows construction of complex two-dimensional patterns as shown in Figure 9. Additional patterns such as bifurcating Y structures, rings,\textsuperscript{49} and cell strands with stepwise increasing widths were readily obtained (data not shown).

**Electrophysiology**

Values for action potential amplitude and maximal diastolic potential reported for heart cells in culture show considerable variability.\textsuperscript{50} Our mean values for maximal diastolic potential (−71 mV) are less negative than those measured in adult rat ventricular tissue (−81 mV)\textsuperscript{51} but are comparable to those reported for ventricles of neonatal rats (−72 mV)\textsuperscript{52} and for monolayer cultures of neonatal rats (−70 mV)\textsuperscript{53,54}. Other authors found less negative membrane potentials in identical cultures (−63 mV\textsuperscript{58}; −60 mV\textsuperscript{56}; −61 mV\textsuperscript{55}). Two reports\textsuperscript{50,56} describe a restoration of maximal diastolic potential depending on culture age. In both of these investigations, MCs initially were depolarized (−49 mV on day 1\textsuperscript{50}; −57 mV on day 3\textsuperscript{56}) and then gradually recovered. In contrast, the cultured MCs in the study of Robinson\textsuperscript{53} and in the present study were normally polarized already on the second day in

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**FIGURE 12.** Column graphs showing action potential duration (upper panel: mean ± SEM of repolarization to 20% of the action potential amplitude; lower panel: mean ± SEM of repolarization to 80% of the action potential amplitude) as a function of culture age and growth pattern: control monolayer (black columns), 65-μm-wide channels (dark gray shaded columns), 100-μm-wide channels (light gray shaded columns), and 150-μm-wide channels (white columns). Numbers above data columns indicate number of analyzed cells. Small asterisks denote significant differences from control monolayer at a given age. Large asterisks above the data columns on day 17 denote significant trends with culture age for a given growth pattern.
culture and subsequently showed no major change in transmembrane potentials. The values for action potential amplitude (93 mV) were lower than those reported for adult rat ventricular tissue (106 mV) by the patterning process itself influencing the shape of the MCs and their degree of differentiation. The method presented allows a great flexibility in the design of MC patterns of interest. Because individual cells contributing to such patterns are readily discernible, the method offers the opportunity to investigate the relation between impulse propagation and structure in heart tissue at the cellular level. Such an investigation, however, would require the application of adequate measurement techniques that permit the mapping of intracellular potentials with a spatial resolution less than or equal to 30 μm and a temporal resolution of approximately 30–50 μsec. Such a technique, based on multiple-site optical recording of transmembrane potentials, is presently under study.

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