Improved Protocol for a Microsphere-Adhesion Assay on Living Tissue Slices

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ABSTRACT

Here, we describe a simple microsphere-adhesion assay to characterize adhesive cues on living tissue slices, which we use to study pattern formation in neural tissue. This assay was developed by modifying a cell-adhesion assay on living tissue slices. We replaced dissociated cells by fluorescent microspheres and then coated the microspheres with isolated membranes from these cells. The membrane-coated microspheres were seeded on living tissue slices, and after a short incubation time, nonadherent microspheres were eliminated by washing. Then, the tissue slices with the adherent microspheres were analyzed using epifluorescence microscopy. As an example, it is shown that membrane-coated adherent microspheres were found to be distributed in a characteristic pattern on living slices of hippocampus, mimicking the adhesion pattern of dissociated living cells. The adhesion assay should be suitable to detect and to analyze adhesive cues on living slices of different tissues and, thus, might have numerous applications in tissue research and developmental studies. Here, we describe and discuss a detailed and improved protocol of the microsphere-adhesion assay.

INTRODUCTION

The adhesive properties of different cell types and the extracellular matrix play an important role in the organization of tissues (4). The use of cell-adhesion assays to study tissue organization has a long history of more than hundred years (9,10,12). The first important findings based on cell-adhesion assays, such as the influence of Ca^{2+} ions on cell adhesion, can be dated to the last century (9,10). Cell-adhesion assays were indispensable for the characterization of the first cell-adhesion molecules (11), and they are important tools to study the role of adhesion molecules in the organization of different tissues (4,11). Cell-adhesion assays on living tissue slices allow for the detection and analysis of tissue-specific adhesive cues (6,7). Recently, we have used a cell-adhesion assay to study the factors that might be involved in the laminar organization of fiber systems in the hippocampus (7). A well-described fiber projection to the hippocampus is the perforant path that originates in the entorhinal cortex and projects with laminar specificity to the outer molecular layer (OML) of the dentate gyrus (2,3). The molecular factors determining this laminar specificity are unknown. To study interactions between cells of the entorhinal cortex and the hippocampus, entorhinal cells were dissociated, fluorescence-labeled and allowed to settle on living slices of hippocampus. When nonadherent cells were eliminated, the remaining cells specifically adhered to the dentate gyrus OML, indicating that the adhesive cues could also contribute to the lamina-specific orientation of growing axons (7). To simplify the analysis of the factors mediating the adhesion on living tissue slices, we replaced dissociated cells by fluorescent microspheres that were coated with isolated membranes from these cells. Adherent membrane-coated microspheres on hippocampal slices were found to be distributed in the same characteristic pattern as the dissociated living cells (7). As a further validation of the microsphere-adhesion assay, we have also analyzed the pattern of adherent membrane-coated microspheres on hippocampal slices of reeler mutant mice. Characteristic malformations of the entorhinal fiber projection are described in the reeler mutant mouse, which lacks the extracellular matrix glycoprotein reelin (13). Again, the distribution of adherent microspheres mimicked the characteristic pattern of entorhinal fibers despite their malpositioning, which indicates that the adhesive cues might be related to the targeting of these fibers (7). Our application for the membrane-coated microspheres is the characterization of the unknown factors involved in mediating the adhesion on living tissue slices. The coating of fluorescent microspheres as a suitable approach to the functional characterization of adhesion molecules was shown previously by coating microspheres with purified known membrane-bound adhesion molecules (5). The microsphere-adhesion assay on living tissue slices described here should have numerous applications in cell and tissue research.

MATERIALS AND METHODS

Preparation of Membrane Suspensions

We have successfully tested two alternative protocols. The first protocol is based on a sucrose-step centrifugation (1). Tissue samples are transferred into a homogenization buffer (10 mM Tris-HCL, pH 7.4, 1 mM spermidine). A cocktail of protease inhibitors (Boehringer Mannheim, Mannheim, Germany) is added immediately before homogenization. Cortices are homogenized with 20 strokes of a dounce homogenizer. The tissue homogenate is then centrifuged in a sucrose-step gradient to remove nuclei and cytoplasm. In a centrifuge tube (1.5-mL polyalomer tube; Beckman Instruments, Fullerton, CA, USA), 350 µL of 50% (wt/vol) sucrose in homogenization buffer are overlaid with 150 µL of 15% (wt/vol) sucrose in homogenization buffer. The tissue homogenate is placed on top and centrifuged at 67 000× g for 10 min at 4°C with a TLS-55 rotor (Beckman Instruments). Nuclei are pelleted, and the supernatant containing water-soluble fractions of the cytoplasm is discarded. The interphase containing the membrane suspension is collected with a pipet and resuspended in 1 mL phosphate-buffered saline.

10 min at 4°C. The suspension is centrifuged again in a TLA 120.2 fixed-angle rotor (Beckman Instruments) at 57,000 × g for 30 min at 4°C. The supernatant is discarded, and the pellet is washed three times in PBS by resuspending and centrifugation with a TLA 120.2 fixed-angle rotor (Beckman Instruments) at 125,000 × g for 60 min at 4°C, and the cell membranes are resuspended. The supernatant is discarded, and the pellet is washed three times in PBS by resuspending and centrifuging with a TLA 120.2 fixed-angle rotor at 57,000 × g for 10 min at 4°C to completely remove the urea. The membranes are resuspended in 1 mL PBS, and then the concentration of the membrane suspension is adjusted to 2 mg/mL by dilution in PBS as described above.

Storage of Membrane Suspensions

Membrane suspensions can be stored for at least several weeks at -20°C. Before freezing, 50% (vol/vol) glycerol is added to the membrane suspension. Before using stored membranes, glycerol is eliminated by centrifugation (Eppendorf® Model 5402 Centrifuge; Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) for 10 min at 20,000 × g, and the membrane pellet is resuspended in PBS.

Coating of Fluorescent Microspheres

Membrane suspensions are diluted and coated on FluoroSpheres® Fluorescent Microspheres (4-µm diameter; Molecular Probes, Eugene, OR, USA) by mixing the microspheres and the membrane suspension, according to the manufacturer’s instructions. Microspheres with different diameters and colors are available from the same manufacturer. As a control, bovine serum albumin (BSA) can be coated on differently stained microspheres.

Preparation of Tissue Slices

Hippocampal slices are prepared according to a standard protocol (7). For the preparation of slices, hippocampi are dissected by using fine spatulas. Hippocampi are sliced perpendicular to their longitudinal axis with a McIlwain tissue chopper (Mickle Laboratory Engineering). Section thickness is 200–400 µm. Slices are placed in a drop of 30 µL incubation medium [50% (vol/vol) Minimal Essential Medium (MEM), 25% (vol/vol) Hank’s Balanced Salt Solution (HBSS), 25% (vol/vol) Heat Inactivated Horse Serum (all from Life Technologies, Paisley, Scotland, UK), 2 mM glutamine and 0.044% sodium bicarbonate (final concentration) adjusted to pH 7.4] in a sterile plastic dish. To distinguish the action of adhesion molecules, which are independent of divalent cations from cation-dependent adhesion molecules, we used either Ca²⁺/Mg²⁺-free HBSS and 1 mM EDTA or, alternatively, PBS (pH 7.4) and 1 mM EDTA as the incubation medium. Both solutions are suitable for adhesion studies in the absence of divalent cations (5,7). Until seeding of labeled-dissociated cells, slices are incubated at 37°C in a humidified 5% CO₂ atmosphere.

Seeding of Membrane-Coated Microspheres on Tissue Slices

Figure 1 schematically describes the seeding of membrane-coated microspheres on the slices. With the aid of a microscope, a few microliters of the microsphere suspension are carefully laid onto the surface of the medium drop covering the slice (Figure 1, Panel 1). The exact volume of the microsphere suspension is adjusted according to the size of the slices. The microspheres are resuspended in the drop and slowly move down towards the tissue slice (Figure 1, Panel 2). After approximately 5 min, the whole tissue slice is covered by the microspheres (Figure 1, Panel 3). Then, the slices are incubated for 30 min at 37°C in 5% CO₂.

Analysis of Slices with Adherent Microspheres

After the incubation, each slice is transferred to a culture dish with a large...
volume of incubation medium using an Eppendorf 1-mL pipet (Eppendorf-Netheler-Hinz-GmbH) (pipet tips are cut to a large diameter with a razor blade). Nonadherent microspheres are removed by gentle shaking of the dish or by carefully pipetting the slice up and down. After the washing procedure, only adherent microspheres remain on the slices (Figure 1, Panel 4). Slices are then mounted on a microscope slide and are analyzed with an Axioskop® microscope (Carl Zeiss Germany, Oberkochen, Germany) equipped for epifluorescence using a 10×, 20× or 40× objective lens. For control, slices are counterstained with the fluorescent dye 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim) to confirm laminar boundaries under UV fluorescence. Differently stained microspheres are simultaneously visible under UV light. As an example, for standardization, red fluorescent microspheres (Catalog No. F8859; Molecular Probes) can be coated with membranes, and yellow fluorescent microspheres (Catalog No. F8858; Molecular Probes) can be coated with BSA (internal standard). Then, the red and yellow microspheres are seeded on DAPI-stained slices in an equimolar ratio. Under UV light, the microspheres can be distinguished by their different color, and their distribution can be analyzed in relation to the pattern of DAPI-stained cells in the tissue slice (7). For permanent preparations, the tissue slices with the adherent microspheres are dried onto micro-

Figure 2. (A) Detail of a living hippocampal slice with adherent membrane-coated microspheres. Microspheres adhere with high density to the OML of the fascia dentata, whereas the inner molecular layer (IML) is repellent for the microspheres. Bar: 30 µm. (B) Detail of a living hippocampal slice with adherent-dissociated, fluorescence-labeled entorhinal cells. Dissociated cells adhere with high density to the OML of the fascia dentata, whereas the IML is repellent for the cells. Bar: 40 µm.
scope slides, covered with a drop of Mowiol® (Hoechst, Frankfurt, Germany) and then covered with a coverslip. Mowiol is used because organic solvents destroy the microspheres.

RESULTS AND DISCUSSION

We have shown that membrane-coated microspheres adhere in a characteristic pattern on slices of hippocampus (Figure 2A), which mimic the pattern of adherent-dissociated cells on hippocampal slices (Figure 2B) and indicate that the same laminar-adhesive cues are recognized by the adherent microspheres (7). In our initial studies, we have prepared membrane suspensions by urea treatment (7). To optimize the adhesion assay, we have tested an alternative protocol for membrane preparations that is based on a sucrose-step centrifugation (see Materials and Methods). Importantly, both protocols were shown to retain the biological activity of membrane-bound molecules in studies on axonal growth (1). Because we achieved identical results with both types of membrane suspensions in the adhesion assay, we recommend to use the sucrose-step centrifugation protocol that substantially simplifies the experimental procedure. The conditions for long-term storage (see Materials and Methods) of membrane suspensions were shown to maintain the biological activity of membrane suspensions on growing nerve fibers for up to several months (1). We have stored membrane suspensions for up to several weeks before use without observing alterations in the adhesion assay. For adhesion studies in the absence of divalent cations, we have used PBS and 1 mM EDTA, as an inexpensive alternative to Ca²⁺/Mg²⁺-free HBSS and 1 mM EDTA, and achieved identical results. Both solutions were shown to be suitable to study Ca²⁺-independent adhesion of protein-coated microspheres (5,7). Before analyzing the distribution of microsphere adhesion on living tissue slices, we recommend to first follow the time course of microsphere adhesion, to determine whether the adhesion pattern changes with increasing incubation time. First, we have tested incubation times ranging from 5 min to several hours. Because we did not observe a time-dependent change of the adhesion pattern on hippocampal slices, we reduced the incubation time for membrane-coated microspheres on the slices from 120 min in our initial experiments (7) to presently 30 min, to save time. Variations of the microsphere-adhesion assay should allow for numerous applications. For standardization for example, the red fluorescent microspheres can be coated with membranes, and the yellow fluorescent microspheres can be coated with BSA (internal standard). The red and yellow microspheres are seeded on DAPI-stained slices in an equimolar ratio. Under UV light, the microspheres can be distinguished by their different color (7). Quantification of microsphere adhesion is easily done by counting the adherent microspheres. Adhesion of membrane-coated microspheres can be tested on slices that were treated with agents known to alter the expression of cell-adhesion molecules. Screening for candidate adhesion molecules can be carried out by coating microspheres with enzyme-treated membranes, membrane subfractions, antibodies, proteins or peptides. Also, slices can be incubated with blocking antibodies against cell-adhesion molecules or treated with specific enzymes before the seeding of the microspheres. Recently, microspheres coated with neurotrophic factors were shown to induce the collateral branching of axons in vitro (8). Similarly, microspheres coated with membranes or membrane fractions should be suitable to study axonal growth.

In summary, we have presented an improved protocol for a microsphere-adhesion assay on living tissue slices. The assay is suitable to detect and analyze adhesive cues on living tissue slices. We suggest that microsphere-adhesion assay has a broad spectrum of applications in histological, cell biological and developmental studies.

REFERENCES


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