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A Simple Method for Measuring Organotypic Tissue Slice Culture Thickness

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Abstract

This paper presents a simple method to measure tissue slice thicknesses using an ohmmeter. The circuit described here is composed of a metal probe, an ohmmeter, a counter electrode, culture medium or physiological buffer, and tissue slice. The probe and the electrode are on opposite interfaces of an organotypic hippocampal slice culture. The circuit closes when the metal probe makes contact with the surface of the tissue slice. The probe position is recorded and compared to its position when it makes contact with the insert membrane on which the tissue grows, thus yielding a thickness measurement. The method does not reduce the viability of slice cultures. Thicknesses of the slice cultures were measured under a number of culturing protocols. An initial drop in thickness occurred between 0 and 4 days in culture. Thicknesses are rather constant thereafter. The type of culture medium and the initial thickness of the tissue explant influence the thickness. Slice thicknesses were compared to a known technique by using optical measurements of slice cross-sections to obtain thicknesses. In contrast to this known technique, the proposed method does not sacrifice the slice culture for measurement purposes. The proposed measurement technique described is straightforward and rapid, about one minute per culture.

Keywords

glia proliferation; hippocampus

1. Introduction

Brain tissue slices and slice cultures are frequently used for neuropharmacological and neurophysiological studies (Baraban and Bausch, 2009). Acute and cultured tissue slices provide easy access for experimental manipulation while maintaining morphology and electrophysiology similar to that *in vivo* (Gahwiler et al., 1997). Freshly excised tissue is sliced to a desired thickness using either a tissue chopper or a vibratome and is used for experiments immediately, or cultured and used later. The overall health (Sykova and Nicholson, 2008), the approach to making certain measurements (Duport et al., 1998; Hamsher et al., 2010; Oldenzel et al., 2006), and diffusion distances (Baraban and Bausch, 2009; Duport et al., 1998; Lipinski, 1992; Robert et al., 1997) are directly correlated to slice thickness, and are pertinent to neurological experiments. This paper presents a simple, direct, and cost-effective method for measuring thicknesses of tissue slice cultures without causing cell death.

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Methods exist for measuring tissue slice thickness; however, those methods either render the tissue slice nonfunctional following the measurement (Stoppini et al., 1991; Takahashi et al., 1995), or submerges the tissue in solution (Lipinski, 1992). In the first case, the slice is cut perpendicular to the long axis of the slice, creating cross-sectional slices, and the thickness is measured optically, as shown in Figure 1. In the second case, double-barreled ion-selective microelectrodes measure steep changes in ion concentration gradients once in contact with the tissue slice (Lipinski, 1992). In the latter procedure, the acutely prepared hippocampal tissue slice is in a bath. The microelectrode detects ion concentration gradients which change significantly at the bath-tissue interface due to the difference between the non-tortuous bath and tortuous tissue. Although a useful technique, translating this technique to slices cultured using the interface method would be cumbersome. In the interface environment, oxygen is supplied from the air above the tissue slice culture and nutrients are supplied from below (Stoppini et al., 1991). Submerging tissue slice cultures may therefore cause hypoxia if the medium is not oxygenated. There is therefore a need for a simple and safe method for determining the thickness of tissue slice cultured by the interface method.

This paper describes experiments using organotypic hippocampal slice cultures that are commonly used for neurological studies (Baraban and Bausch, 2009; Cho et al., 2007; Gahwiler et al., 1997). The hippocampal laminar architecture is preserved, while the biochemistry, synaptic connections, and electrophysiology are similar to those *in vivo* (Baraban and Bausch, 2009). Organotypic cultures survive from weeks to months, providing options for long term studies, such as those studying recovery following excitotoxicity, ischemia, and traumatic injury (Cho et al., 2007). Recently, we have developed a technique to acquire samples of extracellular fluid from these tissue slice cultures based on electroosmotic flow (Hamsher et al., 2010). In this or any other approach to obtain information on the concentrations and distribution of freely diffusing solutes, knowledge of the culture's thickness is essential before, during and after an experiment in the same living slice culture.

2. Materials and methods

2.1 Chemicals and solutions

Two types of culture media were employed: medium A and medium B. Medium A (Guy et al., 2008) consisted of the following obtained from Sigma-Aldrich: 50% basal medium eagle, 25% Earle's balanced salt solution, 23% heat inactivated horse serum, 25 U/mL penicillin-streptomycin, 1 mM L-glutamine, and 41.6 mM D-(+)-glucose. Medium B (Norberg, 2004) was composed of the following materials obtained from Gibco (Invitrogen, Carlsbad, CA): 50% opti-MEM, 25% horse serum, and 25% Hanks' balanced salt solution with phenol red, and 1% D-(+)-glucose (from Sigma-Aldrich, St. Louis, MO). Medium B was filtered through a Nalgene 0.1 μ m polyethersulfone filter (Fisher Scientific, Waltham, MA). The following were also purchased from Sigma-Aldrich unless stated otherwise. Gey's balanced salt solution (GBSS) was supplemented with D-(+)-glucose (27.5 mM) and MgSO_4 (2.7 mM, filtered). HEPES buffered salt solution (HBSS) contained (mM) 143.4 NaCl, 5.0 HEPES, 5.4 KCl, 1.2 MgSO_4 , 1.2 NaH_2PO_4 , 2.0 CaCl_2 , and 10 D-(+)-glucose diluted with Millipore Synthesis A10 system 18 M Ω purified water (Millipore, Billerica, MA). The pH was adjusted to 7.40 using 0.5 mM NaOH solution prior to filtration through a 0.45 μ m nylon Whatman filter (Maidstone, England). Propidium iodide (PI) solution was prepared at a final concentration of 0.35 mM in GBSS. 3% agarose type-V (Sigma-Aldrich) in Hanks' balanced salt solution with phenol red was used for method validation.

2.2 Tissue slice culturing procedure

Organotypic hippocampal slice cultures were prepared as described previously (Guy et al., 2008). The University of Pittsburgh IACUC authorized the following procedures. Hippocampi were excised from seven-day postnatal Sprague-Dawley albino rats. The tissue was cut into slices (at different thicknesses, as indicated) using a McIlwain tissue chopper (The Mickle Laboratory Engineering, Surrey, England). Tissue slices were laid on a porous Millicell insert membrane (Millipore, Billerica, MA) and incubated over medium for up to 14 days in culture.

2.3 Slice thickness measurements

An insert containing one or two tissue slices was placed over culture medium on an inverted microscope (Olympus IX-71, Melville, NY) in order to monitor visually the procedure under bright field. The inset of Figure 1 shows the measurement setup. A 1 mm diameter platinum wire was submerged in the culture medium and connected to a multimeter (Fluka, Everett, WA) to monitor resistance. The other terminal of the multimeter was connected to a 1 mm diameter stainless steel rod. One end of the rod was polished to create a flat surface. The rod was lowered using an electronic micromanipulator (MP-285, Sutter, Novato, CA) until its polished end made contact with either the insert membrane or the tissue. Contact was indicated by the multimeter reading changing from “open circuit”, to less than 16 M Ω , indicating a completed circuit. Contact and rod location were visually confirmed using the microscope. At the point of contact, the resistance and manipulator position were recorded. To determine the standard deviation of the positioning of the micromanipulator, we recorded the position after making contact with the fluid bath, and repeating eight times. Each slice culture thickness was calculated from the difference in the averages of four readings from the surface of the insert membrane readings and four tissue readings. For all tissue measurements, the probe was centered primarily over cell bodies of the trisynaptic loop or over apical dendrites of the pyramidal cells, near the pyramidal cell layer. Twenty-five cultures were measured for day zero in medium A. Otherwise, thicknesses of ten or eleven slice cultures were measured and averaged for each day in culture. All day zero slice culture thicknesses were measured 30 minutes to 2 hours following explantation.

To compare the proposed method to an existing method of measuring thickness, 5 (400 μ m initial slice thickness) and 3 (350 μ m initial slice thickness) were cultured for 5 days. On day 5 in culture, each of the slice culture thicknesses was measured using the probe. The slices were divided into 3 groups. The first group of slice cultures (N = 3 slice cultures) was separated from any excess insert membrane, and the slice cultures were cut at 600 μ m spacing using the tissue chopper, see Figure 1. Each slice culture yielded approximately 4 cross-sectional slices that contained at least one of the following regions: dentate gyrus, CA1-CA4, or the subiculum. The cross-sectional slices were set on a coverslip along the freshly sliced edge and imaged over the IX-71 microscope under bright field using an Olympus U Plan Apo 4 $\times$ objective lens. Additionally, an Olympus Plan Apo 1.25 $\times$ objective lens was used for the bright field image in Figure 1. The second group of slice cultures (N = 3 slice cultures) were fixed using 4% paraformaldehyde in PBS (Boston Bioproducts, Ashland, MA) for 1 hour, after which the tissue cultures were cut into cross-sectional slices and imaged as in the first group. Slice cultures in the third group (N = 2 slice cultures) were cut, then fixed in paraformaldehyde and imaged. Each image yielded 3 to 8 thickness measurement and measurements of the insert membrane thickness. The thickness measurements of all the cross-sections of the same slice culture were averaged and compared to the measured thickness of the same tissue culture using the probe method.

2.4 Viability measurements

Thirty slice cultures were randomly selected to check if the process of measuring slice thickness compromised viability. Following the thickness measurements, medium was exchanged with fresh 37 °C medium supplemented with PI (final concentration: 7 µM). Following a 24 hour incubation period, the medium was exchanged with 37 °C GBSS and incubated for 30 minutes twice and 37 °C HBSS a third time. Negative control slice cultures followed the same exchange procedure. The negative control slices remained in the incubator following exchanges. To serve as a set of positive controls, a drop of methanol was placed on top of slice cultures prior to PI exposure. Methanol ensures cell death of both glia and neurons throughout the slice culture (Hamsher et al., 2010). PI fluorescence indicating cell death was imaged using a U-MGIW2 cube (Olympus) and Simple PCI software (Compix, Cranberry, PA). Exposure times were set according to the fluorescence intensity of the positive controls. Analysis of slice culture images were done with Simple PCI. Average intensities of regions of interest encompassing the laminar structure of the slice cultures were collected and analyzed using Equation 1 (Hamsher et al., 2010). The intensity results for all the controls were averaged within their given treatments, resulting in an average PI intensity for the specific day in culture.

$$\% \text{Cell Death} = \frac{\text{PI intensity (experimental)} - \text{Average PI intensity (negative control)}}{\text{Average PI intensity (positive control)} - \text{Average PI intensity (negative control)}} \times 100\% \quad (1)$$

2.5 Method validation

Agarose gel (3% w/v) was cut to approximately 3.5 mm thick slab. Attempts at creating gel slabs of similar thicknesses as the slice cultures proved unfeasible, yielding uneven slabs. The 3.5 mm thick gel was laid on its end on a coverslip over the microscope and was imaged in bright field. Several thickness measurements were made at different points along the length of the slab. The procedure was repeated for the same gel slab on the opposite face. The gel was then laid on an insert membrane over HBSS, like the tissue setup. The thickness of the slab was measured by averaging manipulator readings from 4 points of contact with the steel rod, and subtracting from the readings of 4 points of contact with the membrane. Once complete, two sides of the slab were again imaged and measured using Simple PCI software on a coverslip.

3. Results

3.1 Slice culture thickness

The standard deviation in the positioning of the micromanipulator is 14 µm (N=8). To insure that the measurement error is approximately 10 µm, four repeat measurements were made on the insert membrane surface and four were made on the tissue. Figure 2 shows the average thicknesses of slice cultures for three different initial slice thicknesses (set by the tissue chopper) and two different culture media. See supplementary content for a table of values corresponding to Figure 2. Day 0 slice culture thicknesses were measured 30 minutes to 2 hours following explantation. Slices cultured in medium A were measured every day in culture from day 0, directly after tissue extraction, until day 14. The other points represent slices cultured in medium B with different initial slice thicknesses. Slice culture thickness levels off between days 4 and 14, during which time the average tissue thickness is 158 ± 3 µm (SEM) for medium A with an initial slice thickness of 500 µm. Medium B produces average slice thicknesses for days 4 – 14 in culture of (mean ± SEM) 155 ± 4 , 182 ± 4 , and 192 ± 3 µm, with initial slice thicknesses of 300, 400, and 500 µm, respectively. The two different culture media result in statistically different slice culture thicknesses over this time

period, with $p < 0.0001$. Independent single-factor ANOVA of 500 μm initial slice thickness cultured over the two media shows that there is a slight dependence of thickness on the day in culture. Two-factor ANOVA shows that initial slice thickness and the day in culture affects the thicknesses of slices cultured in medium B ($p < 4 \times 10^{-3}$).

3.2 Viability

Minimal cell death of $3.9 \pm 1.2\%$ in the slice cultures is incurred as a result of thickness measurements using the probe as compared to negative and positive controls.

3.3 Method validation

A gel slab was used to validate the proposed measurement method. Images of the gel slab taken before probe thickness measurements resulted in a thickness of $3.44 \pm 0.03\text{ mm}$. The slab thickness measured using the proposed probe technique was $3.51 \pm 0.05\text{ mm}$. The thickness measurement of the slab using image analysis following the probe measurements was $3.52 \pm 0.02\text{ mm}$. Single-factor ANOVA confirms that the probe measured thickness is statistically the same as the image analysis measurements ($p > 0.05$).

3.4 Method comparison

A paired t-test reveals that the proposed method yielded thicknesses that are $40 \pm 9\text{ }\mu\text{m}$ ($N = 8$) thicker ($p \leq 0.004$) than when the same slice cultures were cut into cross-sectional slices as shown in Figure 1 and measured in bright field images.

4. Discussion

Figure 2 shows a significant decrease in slice culture thickness within the first few days. Measurements taken within a couple of hours following explantation show that the slice thickness is 20% (medium B) to 30% (medium A) less than the chopper setting. There is no observable dependence of slice culture thickness on time within the 30 minutes to 2 hour time-span following explantation. These results show thinning occurring on a shorter timescale compared to Stoppini's original estimation of one week (Stoppini et al., 1991). The two media produce slightly different slice thicknesses once the slice thicknesses level off. The different thicknesses may arise from differences in glial cell proliferation (Holopainen, 2005; Muller et al., 2001). Once in culture, reactive astrocytes blanket the surface of the sectioned tissue slice (Cho et al., 2007; Muller et al., 2001). Varying rates of glial cell proliferation (Gahwiler et al., 1997) and cell differentiation could also explain the variation in thicknesses among slice cultures. Other factors affecting slice thickness are related to surgery, such as variations in time preparing the slice culture immediately after explantation.

Current methods available for measuring slice culture thickness have significant drawbacks for consecutive measurements in individual living tissues. We found measuring slice thickness using cross-sectional slices unreliable. The slice cultures were consistently measured thinner once chopped compared to when measured with the probe. The primary concern relates to "pinching" of the slice culture along the chopped edges. Furthermore, manipulation of the cross-sectional slices is challenging. The cross-sectional slice has a tendency to dry out or not lay perfectly perpendicular to the coverslip. As stated earlier, the use of ion-selective microelectrodes is cumbersome, and therefore was not used as a method of validation.

The use of a 3% gel slab allows for optical measurements without risking dehydration of the gel. The gel consists of Hanks' balanced salt solution, such that the multimeter responds when the stainless steel probe makes contact with the surface of the gel. Attempts at cutting gel to a thickness similar to that of tissue slices resulted in variations in the slab thickness.

Larger slabs proved to have a more level surface. Optical thickness measurements confirmed thickness measurements made with the stainless steel probe, thus validating the proposed method of thickness measurements.

Measuring slice thickness is effective for screening and characterizing brain slice cultures prior to use in experiments. Brain tissue slices or cultures that are of thicknesses other than expected are likely unhealthy tissues (Sykova and Nicholson, 2008). The technique can be useful in real time consecutive measurements of tissue swelling under various conditions over days and weeks in the same tissue slice, as opposed to final characterization methods requiring sacrifice of the slice culture (Stoppini et al., 1991; Takahashi et al., 1995). Characterizing slice cultures prior to experiments also allows for proper placement of sampling and measurement probes and capillaries (Hamsher et al., 2010). Furthermore, sampling analytes from the extracellular space is affected by slice thickness (Duport et al., 1998; Hamsher et al., 2010; Robert et al., 1997). Variations in thicknesses among the slice cultures strengthen the argument that slice culture thickness should be measured.

The method proposed in this paper is easy, effective, expeditious, and inexpensive. It causes negligible cell death, and does not require the slice cultures to be submerged in a bath. Furthermore, none of the components are fragile. This simple technique may be applied for characterization and screening of any tissue slice or culture that is not submerged in solution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) A micrograph of the slice culture (day 5 in culture) at a magnification of 125 $\times$. To measure the thickness using the cross-section of the slice, the insert membrane was cut around the slice culture approximately along the indicated rectangular line. The slice was then cut in the direction of the white dotted line to yield approximately 4 cross-sectional slices spaced 600 μ m apart. B) A micrograph of the same slice culture at 400 $\times$ magnification. C) A micrograph of a cross-sectional slice. The thickness was measured as the height of the cross-section without the insert membrane.

Figure 2.

The average thicknesses on the specified days in culture and the standard errors of the mean, SEM. Purple exes represent medium A tissue slices with an initial slice thickness of 500 μm . Red squares represent medium B and 300 μm initial slice thickness. The blue diamonds represent medium B and 400 μm . The green triangles represent medium B and 500 μm . Inset: The setup of the measurement circuit.