Video Article

In Vivo Microdialysis Method to Collect Large Extracellular Proteins from Brain Interstitial Fluid with High-molecular Weight Cut-off Probes

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Abstract

In vivo microdialysis is a powerful technique to collect ISF from awake, freely-behaving animals based on a dialysis principle. While microdialysis is an established method that measures relatively small molecules including amino acids or neurotransmitters, it has been recently used to also assess dynamics of larger molecules in ISF using probes with high molecular weight cut off membranes. Upon using such probes, microdialysis has to be run in a push-pull mode to avoid pressure accumulated inside of the probes. This article provides step-by-step protocols including stereotaxic surgery and how to set up microdialysis lines to collect proteins from ISF. During microdialysis, drugs can be administered either systemically or by direct infusion into ISF. Reverse microdialysis is a technique to directly infuse compounds into ISF. Inclusion of drugs in the microdialysis perfusion buffer allows them to diffuse into ISF through the probes while simultaneously collecting ISF. By measuring tau protein as an example, the author shows how its levels are altered upon stimulating neuronal activity by reverse microdialysis of picrotoxin. Advantages and limitations of microdialysis are described along with the extended application by combining other *in vivo* methods.

Video Link

The video component of this article can be found at https://www.jove.com/video/57869/

Introduction

ISF comprises 15-20% of total brain volume and offers a microenvironment critical for signal transduction, substrate transport and waste clearance[1]. Therefore, the ability of collecting ISF from living animals will provide greater implications for various biological processes as well as disease mechanism. *In vivo* microdialysis is one of the few methods that sample and quantify extracellular molecules from ISF from awake, freely moving animals and thereby serves as a useful tool in neuroscience research field[2,3]. In this method, microdialysis probes with semipermeable membranes are inserted in the brain and perfused with perfusion buffer at the relatively slow flow rate (0.1-5 µL/min). During this perfusion, extracellular molecules in ISF passively diffuse into the probe according to the concentration gradient and collect as a dialysate. Although this article focuses on the method to sample ISF in the brain, both the principle and the method can be applied to other organs by appropriate modification if necessary.

Microdialysis was first employed in the early 1960s, and since then it has been extensively used to collect small molecules including amino acids or neurotransmitters in brain. However, recent commercial availability of microdialysis probes with high-molecular weight cut off membranes (100 kDa-3 MDa) has extended its application to relatively larger proteins in ISF as well[4,5,6,7]. The studies using these probes has led to the finding that proteins such as tau or α-synuclein that were long thought to be exclusive cytoplasmic are also physiologically present in ISF[4,5,8].

One of the difficulties using microdialysis probes with large cut off membranes (typically over 1,000 kDa) is that they are more susceptible to ultrafiltration fluid loss due to the inner pressure accumulated in the probes. Microdialysis probes used here have a unique structure to avoid this issue. The pressure will not be built up due to this structure, thus microdialysis with these probes should be operated in a "push-pull" mode using a syringe pump to perfuse the probes (=push) and a roller/peristaltic pump to collect the dialysate coming from the probe outlet (=pull)[9] (Although it needs both push and pull pumps, due to pressure cancelling vent holes present in the probes, the system is technically only driven by the pull pump). This article starts with the stereotaxic surgery of a guide cannula implantation and describes how to set up microdialysis lines in order to collect ISF through microdialysis probes with 1,000 kDa cut-off membranes.

Protocol

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Graduate School of Medicine at the University of Tokyo.

1. Pre-surgical Procedure

1. Before starting surgery, wipe everything with 70% ethanol to maintain sterile conditions. Thermal support using a heating pad is recommended.
2. Anesthetize the mice by intraperitoneal injection of chloral hydrate (400 mg/kg). Confirm anesthetization by performing a toe pinch. Use of meloxicam SR at induction and buprenorphine upon recovery BID for at least 24 h is recommended.
3. Shave the hair with a surgical clipper. Fix a mouse in the mouse and neonatal rat adaptor using ear bars and a nose clamp.
   NOTE: It is critical to make sure that mouse head is secured at this stage and does not move side-by-side.
4. Apply vet ointment on eyes to prevent dryness while under anesthesia.

2. Stereotaxic Surgery for Guide Cannula Implantation

1. Set the mouse and neonatal rat adaptor on the stereotaxic apparatus. Make an incision sagittally on the skin over the skull using a scalpel. #Wipe off blood and the connective tissue on the skull using a damp cotton swab.
2. Determine the coordinate for brain region of interest using brain atlas. Before starting measurements, make sure that midline is straight so that the drill bit can be moved A-P and remain on the midline suture the entire time.
   NOTE: This article uses the coordinate (A/P: -3.1 mm, M/L: -2.5 mm, D/V: -1.2 mm, 12 degrees) to target posterior hippocampus.
3. Level skull A-P
   1. Attach a drill on a manipulator of stereotaxic frame.Lower the drill until it gently touches lambda and record its ventral coordinate. Repeat this procedure for bregma.
      NOTE: When the skull is leveled, the vertical measurement of bregma is equal to that of lambda. If not, adjust the height of nose clamp accordingly. After the skull gets leveled, record the anterior/posterior, lateral coordinate of bregma.

4. Level skull left-right
   1. Move the drill from bregma to the coordinate (A/P: -3.1 mm, M/L: +2.0 mm), lower the drill to the skull and record the vertical coordinate. Then repeat this procedure for the coordinate (A/P: -3.1 mm, M/L: -2.0 mm).
      NOTE: If the skull is leveled, these vertical measurements of two equidistant points from the midline are equal. If not, adjust the height of the ear bars.

5. Drill a burr hole carefully at the target coordinate (A/P: -3.1 mm, M/L: -2.5 mm) to implant a guide cannula. If the diameter of a burr hole is not large enough for a guide cannula implantation, drill another hole that overlaps with the first one. Drill another hole on the right (contralateral side) parietal bone and insert a bone screw, which helps to secure dental cement in 1.10 (See **Figure 1B**).
6. Cut a circular locking piece from the back side of lid of a 1.5 mL centrifuge tube by a razor blade and make a "crown". This crown is used to prevent dental cement from spreading to the skin. Place it on the skull so that the burr holes made in step 2.5 stay within the circle (See **Figure 1**).
7. Set up a stereotaxic assembly by putting a guide cannula on the shorter arm of a stereotaxic adaptor and fasten it using a cap nut. Set the longer arm of the stereotaxic adaptor on the electrode clamp. Attach it on the manipulator of the stereotaxic apparatus (See **Figure 1A**).
8. Rotate the D-V stereotax assembly on the manipulator arm by 12 degrees (See **Figure 1B**). Move the guide cannula to the burr hole made in 1.6. Insert the guide cannula slowly into the brain by lowering it by 1.2 mm.
   NOTE: The angle of the probe is specific to the hippocampus; other regions may require other angles or no angle at all. Consult a brain atlas for precise coordinates. Dry the surface of the skull, because if not the cement will not stick and cement cap can become dislodged
9. Add the dental cement within the crown to fully cover both the metal part of the guide cannula and the bone screw enough to secure them. Apply additional dental cement if there is a part of skull exposed.
10. Wait until dental cement is completely dried (~12-20 min). Remove the stereotaxic adaptor from the electrode clamp. Remove the cap nut and replace the stereotaxic adaptor with a dummy probe and fasten the cap nut.
11. Release the mouse from the stereotaxic apparatus and house the mouse alone in an individual cage.
    NOTE: The mouse should not be left unattended until it has regained sufficient consciousness to maintain sternal recumbency. Check the mouse daily until the day of microdialysis. The mouse receives NSAIDs such as carprofen if it appears to be in pain. Waiting 2 weeks is necessary for sleep-wake studies so the mouse habituates to the new environment[10], but other types of studies may require shorter recovery periods (*e.g.,* 1-2 days).

3. Microdialysis Setup

1. Quality-check of probes: Fill a disposable 1ml syringe with distilled waterand connect it to the outlet (shorter port) of a probe using a byton tube. Cover the vent holes with the fingers and depress the syringe plunger gently to infuse water to the probe. Check that water appears from the probe inlet and there is no leakage on the surface of a microdialysis membrane.
   1. Activation of probes: Submerge the membranes of a probe in ethanol (70-100%) for two seconds. Then, infuse distilled water into the probe again with a syringe again.

2. Preparation of perfusion buffer: In order to avoid adhesion of target molecules to the tubings,add BSA by diluting 30% BSA solution to 4% with artificial CSF (1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 3 mM KCl, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 122 mM NaCl, pH=7.35), which closely matches electrolyte concentration in CSF, immediately prior to use. Filter the perfusion buffer through a syringe filter unit with 0.1 µm pore size .
   NOTE: 4% BSA improves recovery for sticking proteins but can severely limit delivery of compounds, especially for compounds that have high BSA-binding. This is one advantage of using lower concentration of BSA (such as 0.15%) in certain instances[3]. Note that BSA can aggregate very easily when agitated by vortex or stir plate. These aggregates can clog probes or membrane pores. Use caution when preparing a BSA solution to limit this aggregation

3. Prepare two separate lines for inlet and outlet (See **Figure 1C**) and connect both lines with a connection needle. Fill a disposable 3 mL syringe filled with perfusion buffer and connect it with the inlet end of the tubing using a blunt-end needle. Fill the entire tubing with perfusion buffer by running the syringe pump.
4. Stop the syringe pump and replace the connection needle between inlet line and outlet line with an activated microdialysis probe from step 3.3 (See **Figure 1C**). Before this replacement, put the cap nut on the probe.
5. Mount the roller tube in the outlet tubing on the roller pump. Start the syringe pump at 10 µL/min and then the roller pump at the slightly slower flow rate (9.5-9.8 µL/min). Make sure to remove all air bubbles in the entire tubing, which may influence the recovery when they enter the probe.
6. Anesthetize the mouse from step 1.12 in the same way as in step 1.2. Put the mouse collar around its neck. Remove the cap nut and dummy probe and slowly insert a microdialysis probe from 3.4 through the guide cannula and fasten the cap nut.
7. Place the mouse in the cage connected to a free-moving system and tether the mouse with the collar. Keep running both syringe pump and roller pump at the indicated flow rate in step 3.5 for at least 1 h.
   NOTE: To achieve ISF collection from awake animals, this article uses a free-moving system, where the cage itself responds to an animal's movement to keep tubings from twisting. Alternatively, liquid swivels available from various companies can be also used.
8. Stop the roller pump first and then the syringe pump. Set the desired flow rate. Run the syringe pump 20% faster than the roller pump.
   NOTE: For instance, if you run at 1 µL/min, operate the syringe pump at 1.2 µL/min. Optimal flow rate should be determined empirically for each molecule.
9. Collecting ISF samples: place the free end of outlet tubing on the refrigerated fraction collector.
   NOTE: Appropriate sample volume varies depending on assays used for analysis.
10. After the completion of the experiment, remove the probe. (Anesthetize the mouse as needed.) Handle mouse recovery the same way as in step 2.11. Analyze the collected ISF by methods such as HPLC or ELISA.
11. To wash the entire tubing after microdialysis, connect inlet and outlet tubings again by replacing the probe with the connection needle and run the diluted bleach in the entire tubing and then flush it with water. Dry and store it for repeated use.
    NOTE: Other types of tubings are acceptable, however, BSA in perfusion buffer can clog the tubings with small diameter, thus these tubings are considered as single-use. The tubings can be worn down or clogged after multiple uses, so make sure that the flow rate is consistent every time before use.

Representative Results

To stimulate or inhibit neuronal activity in reverse microdialysis[11,12,13], picrotoxin, $GABA_A$ receptor antagonist or tetrodotoxin, Na^+ channel blocker have been used. It has been shown that tau release is stimulated by increase of neuronal activity[13,14]. Consistent with these previous observations, when 50 µM picrotoxin (PTX) was administered via reverse microdialysis (see Discussion for more details) in awake C57B6/J mice increased ISF endogenous tau levels measured by ELISA compared to vehicle control (DMSO) (**Figure 2**).

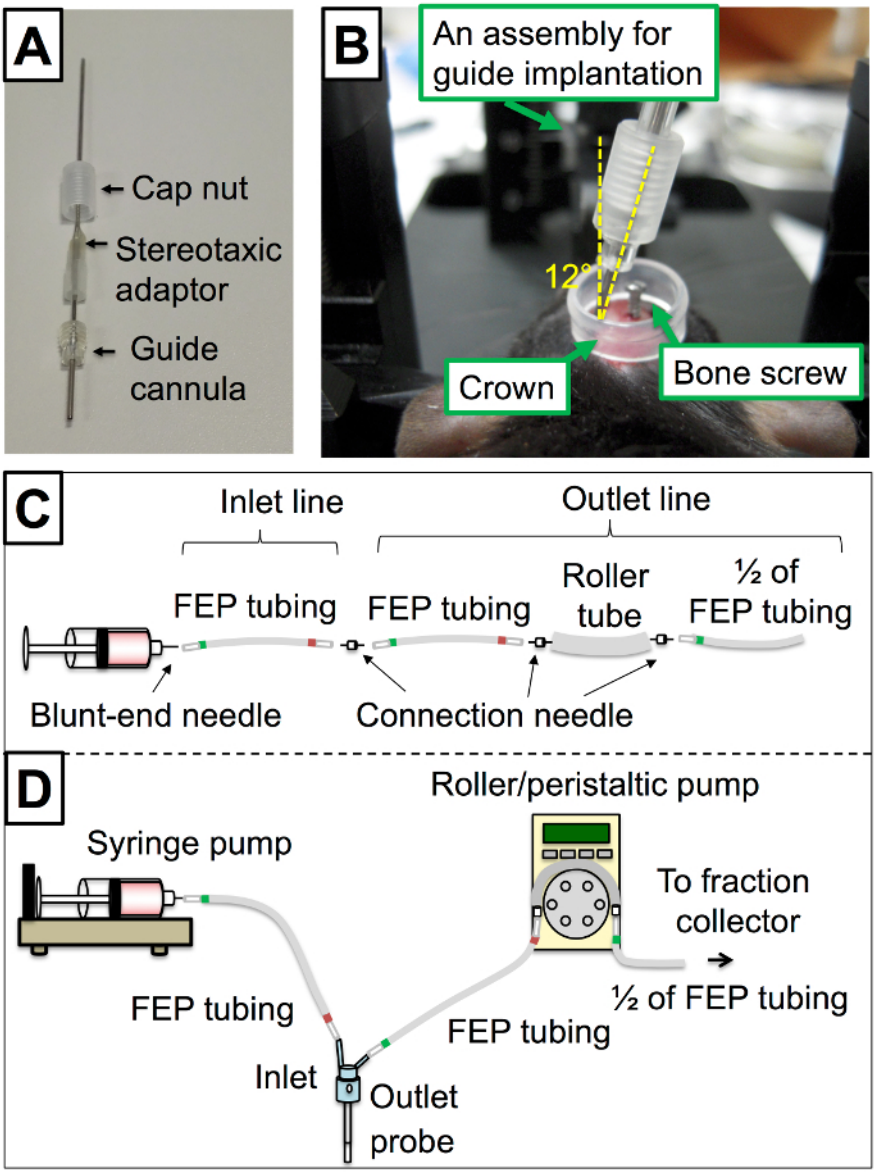

Figure 1: Surgery setup and the construction of microdialysis circuits. (A) Set up a stereotaxic assembly for a guide implantation using a cap nut, a stereotaxic adaptor and a guide cannula. **(B)** Stereotaxic surgery to implant a guide cannula. The guide cannula is inserted in the brain at 12 degrees with respect to vertical. **(C)** Construction of an inlet tubing and an outlet tubing. Inlet line consists of 70 cm FEP tubing (JF-70) and the outlet line consists of JF-70 and the roller tube and 1/2 of FEP tubing. The connection needles are used for each connection. **(D)** Once the entire tubing is filled with perfusion buffer, stop the pump and replace the connection needle between inlet line and outlet line with an activated microdialysis probe. Make sure to connect the end of inlet tubing to the inlet port of a probe (longer port) and connect the end of outlet tubing with outlet port of a probe (shorter port). Please click here to view a larger version of this figure.

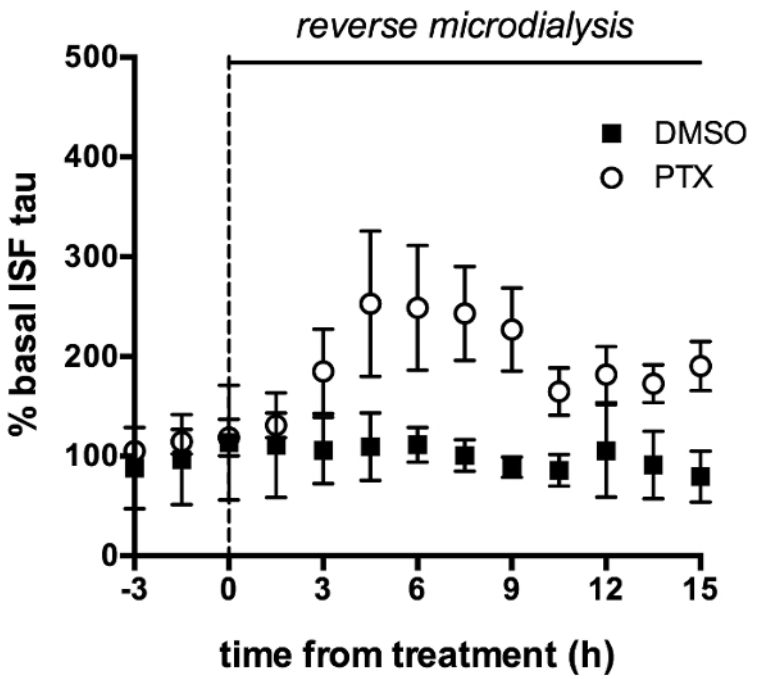

Figure 2: Representative data showing ISF tau changes upon picrotoxin reverse microdialysis. Picrotoxin (PTX, 50 µM) or DMSO were delivered into the hippocampus of C57B6/J mice via reverse microdialysis. Picrotoxin rapidly increased ISF endogenous tau levels from its basal levels (*i.e.,* the average tau concentrations during 3 h before PTX administration) compared to DMSO treatment. Data is shown as mean±SEM, n = 3 for DMSO, n = 4 for PTX. Please click here to view a larger version of this figure.

Discussion

Microdialysis with high molecular weight cut off membranes has to be operated by a push-pull mode, thus it is critical that the flow rate is accurate and constant. The inaccuracy in the flow rates can be the cause of air bubble generation and inconsistency in the sample concentration. If the flow is inconsistent, check all connections for leakage. If the problem still persists, it may be necessary to re-start with new probes and tubings.

Microdilaysis probes are continuously perfused by the perfusion buffer. Therefore, there is not sufficient time for extracellular molecules to reach a full equilibrium in the perfusion buffer. As a consequence, the concentration in the dialysate is much lower than its actual concentration in ISF. In order to estimate the true concentration of molecules in ISF, a zero flow method is often used[3,5,15]. This method measures the concentration by altering the flow rate. There is an inverse relationship between the concentration and the flow rate. Therefore, the concentration of target molecules can be determined by extrapolating the exponential curve back to the theoretical zero flow, which represents the perfect recovery of the molecules. However, the recovery can be influenced by various factors such as interaction with membranes or hydrophobicity of molecules or interaction with other proteins, and one should keep in mind that the concentration at the theoretical zero may not be necessarily equivalent to its true concentration in ISF.

A probe insertion causes acute local injury in brain. From this reason, the first several fractions are typically excluded from further analysis. In fact, the levels of ISF tau protein in hippocampus are not stable in the first 9-15 hour likely because the acute injury causes non-specific release of tau into ISF (the author's unpublished observation). Recovery after probe insertion injury varies from target to target. Pilot studies should be performed to determine when each target reaches a steady-state level to determine when samples should be analyzed. This determines the "start point" for microdialysis sampling. An endpoint for sampling is when the target is no longer at the steady state (typically 3-5 days after microdialysis probe implantation; not guide implantation). Start and end for each target should be determined empirically by each lab for each target.

Microdialysis is especially useful when combined with drug treatment. Drugs can be administered systemically or directly infused into ISF. Reverse microdialysis is one method that directly infuses small compounds via probes. Dialysis occurs bi-directionally. Therefore, inclusion of drugs in the perfusion buffer allows its diffusion though microdialysis probes to the surrounding tissue. This method enabled the local administration of small compound and simultaneous collection of ISF. Reverse microdialysis is less invasive compared to a pressure injection through cannula and can maintain more constant concentration of drugs in target area.

Before starting reverse microdialysis, the internal volume of entire tubing should be taken into account to calculate the timing of treatment and sample collection. For example, the internal volume of entire tubing used in this article is 91.5 µL (Refer to the **Table of Materials** for internal volume of each tubing). Therefore, when the flow rate is 1.0 µL/min, it takes 91.5 min for the perfusion buffer containing drugs to reach the fraction collector. On the other hand, when drugs are delivered systemically, the inner volume of the outlet tubing (56.5 µL in this case) should be considered.

In vivo microdialysis method offers several advantages over other techniques. For example, while brain homogenates likely consist of both extracellular and intracellular proteins, microdialysis specifically collects extracellular proteins from ISF. Second, a single microdialysis probe can collect ISF at the different time points from the same animal. Target concentration in each sample can be normalized to a % baseline. This normalizes absolute concentration between animals so relative differences can be compared. This increases power of a study and typically reduces animal number needed for an experiment when comparing relative differences in protein levels following drug administration/ manipulation. On the other hand, the major limitation of microdialysis is the restriction of the size of the target molecule.

Microdialysis can be combined with other *in vivo* techniques. For example, performing microdialysis in regulatable transgenic mice can estimate *in vivo* turnover of target protein[16]. The combination with EEG, measurement was used to measure electric activity during reverse microdialysis[11]. And optogenetics enabled manipulation of neuronal activity in brain while simultaneously collecting ISF[17].

Disclosures

The author has nothing to disclose.

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