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Fluorescein isothiocyanate (FITC)-Dextran Extravasation as a Measure of Blood-Brain Barrier Permeability

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Abstract

The blood-brain barrier (BBB) is formed in part by vascular endothelial cells that constitute the capillaries and microvessels of the brain. The function of this barrier is to maintain homeostasis within the brain microenvironment and buffer the brain from changes in the periphery. A dysfunction of the BBB would permit circulating molecules and pathogens typically restricted to the periphery to enter the brain and interfere with normal brain function. As increased permeability of the BBB is associated with several neuropathologies, it is important to have a reliable and sensitive method that determines BBB permeability and the degree of BBB disruption. A detailed protocol is presented for assessing the integrity of the BBB by transcardial perfusion of a 10,000 Da FITC labeled dextran molecule and its visualization to determine the degree of extravasation from brain microvessels.

Keywords

Blood-brain barrier; FITC-dextran extravasation; brain capillaries

INTRODUCTION

The brain has a network of capillaries that delivers nutrients and oxygen and removes waste products generated by neurons and glia (Abbott et al., 2006). This neurovascular unit includes capillaries that are composed of tightly aligned vascular endothelial cells that strictly regulate the entrance of large hydrophilic molecules into the brain. The physicochemical barrier that is formed is referred to as the blood-brain barrier (BBB) (Risau, 1996). Many disease conditions including stroke, epilepsy, Multiple Sclerosis, Alzheimer's disease, human immunodeficiency virus, traumatic brain injury, and drug abuse

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damage the BBB (Chodobski et al., 2011; Dallasta et al., 1999; Marchi et al., 2012; Marques et al., 2013; Minagar and Alexander, 2003; Northrop and Yamamoto, 2015; Sandoval and Witt, 2008). Therefore, a reliable method is needed to study whether and to what extent the BBB is permeable in order to determine the degree of disruption and to devise appropriate therapeutic strategies.

Agents such as Evans Blue, albumin, horseradish peroxidase (HRP), immunoglobulin (IgG), radioactive tracers, sodium fluorescein and dextran have been typically employed to assess BBB permeability (Saunders et al., 2015). The choice of the appropriate molecule is important when assessing BBB permeability such that a good marker of BBB integrity should be inert and without any effect on the physiology and function of the animal. Additionally, the marker should be large enough to remain in healthy brain capillaries and be able to freely diffuse out of compromised blood vessels. Evans Blue relies on its conjugation with albumin to form a large molecule. The drawback is that smaller unbound Evans Blue molecules can still diffuse out of intact capillaries and enter the brain parenchyma without significant increases in BBB permeability, leading to false positive conclusions. IgG is a very large molecule and may not reveal subtle disruptions in the BBB. In addition, since IgG and similarly, albumin is endogenous to the body and present in the blood at all times, the exact onset of BBB disruption would not be apparent by measuring IgG or albumin in the brain. Thus, it is important to use different markers that can reliably assess variations in timing and permeability of the BBB as the opening of the BBB is not an all or none phenomenon, can occur in a graded manner, and can be the cause or the consequence of another event.

One marker is fluorescein isothiocyanate (FITC) labeled dextrans. FITC-dextrans are polysaccharides comprised of varying lengths of branched glucose molecules (Hoffmann et al., 2011). These molecules are commercially available in molecular weights ranging from 4 – 70 kDa and can be used to determine solute, ion and protein permeability of the BBB based on the size of the dextran used. Because FITC-dextrans are exogenous and can be administered at discrete intervals, in contrast to endogenous molecules like IgG and albumin, they can provide valuable information on the time course and duration of BBB permeabilization. An added benefit of FITC-dextran is that low brain concentrations of fluorescein can be measured due to the high sensitivity of fluorescence detection and its negligible binding to plasma proteins (Wolman, 1981).

BASIC PROTOCOL 1: Assessment of FITC-dextran extravasation from the BBB

The protocol below details the methodology for perfusing 10,000 Da FITC-dextran in anesthetized Sprague-Dawley rats to assess BBB integrity. While Sprague-Dawley rats were used, this procedure could be adapted for other rodents, and possibly other non-primates, if body weight is accounted for. Once FITC-dextran has been perfused it fills brain capillaries and can be observed using a fluorescence microscope or under excitation by the 488 laser. The brain capillaries and surrounding parenchyma with FITC-dextran can then be imaged and analyzed to assess BBB integrity.

All experiments were conducted in accordance to National Institute of Health Guide for Care and Use of Laboratory Animals and approved by Indiana University Institutional Animal Care and Use Committee.

Materials

- 0.1M and 0.2M Phosphate Buffered Saline (PBS) (see recipe)
- 4% paraformaldehyde (PFA) solution (see recipe)
- 10% and 20% Glycerol solution (see recipe)
- FITC-dextran solution (see recipe)
- Gelatin subbing solution (see recipe)
- Brain freezing solution (see recipe)
- Millipore™ water
- 250–300 g Sprague-Dawley rats (Harlan, RRID:RGD_10395233)
- Ketamine
- Xylazine (Butler Schein cat. no. 33197)
- Heparin
- 26 G needle (Fisher scientific, cat. no. 14-826-10)
- 16 G11/2 needle (Fisher scientific, cat. no. 14-821-14H)
- 1 mL syringe (Fisher scientific, cat. no. 14-823-434)
- Perfusion pump (Fisher scientific, cat. no. 13-876-2)
- Blunt scissors (Fisher scientific, cat. no. 08-950)
- Micro dissection scissors (Fisher scientific, cat. no. 08-935)
- Hemostat (Fisher scientific, cat. no. 16-100-118)
- Bone rongeurs (Fisher scientific, cat. no. NC0688492)
- Guillotine (Harvard Instruments cat. no. 55-0020)
- Spatula (Fisher scientific, cat. no. S50823)
- Glass beaker (Fisher scientific, cat. no 02-540K, 02-540T))
- Graduated cylinder (Fisher scientific, cat no. 03-007-45)
- Filtering apparatus (Duran cat. no. 2134031)
- Filter paper (Sigma cat. no.58060-U)
- Stir bar (Fisher scientific, cat. no. 14-513-51)
- Cryostat (Fisher scientific, Cryostar NX50)
- Pedestals (Fisher scientific, cat. no. 715870-CN)

- TissueTek (Electron microscopy sciences, cat. no. 62550-12)
- Glass slides (Worldwide medical, cat. no. 48011066)
- Slide racks (Wheaton, cat.no. 900254)
- Coverslip (Fisher scientific, cat.no. 12-545-100)
- Slide box (Fisher scientific, cat.no. 03-446)
- Small sample jars (Weaton, cat.no. W216971)
- Fluoromount-G (Electron microscopy sciences, cat. no. 17984-25)
- Ice bucket (Fisher scientific, cat. no. 07-210-129)
- DRAQ5 (Cell signaling, cat.no. 4054S, RRID:AB_2314341)
- Hot plate (Fisher scientific, cat.no. 11-497-6A)
- Confocal microscope with 488, 633 excitation laser
- Image J software (http://rsb.info.nih.gov/ij/index.html, RRID:SCR_003070)
- Microfiber cloth
- Aluminum foil
- Slide rack (EMS, cat.no. 71394-01)
- Deep tray for submerging slide rack (12″L X 6″W X 2″D)
- Dry ice
- Oven (Norco Model 410)
- Access to sink and water

Protocol steps—Step annotations

Perfusion pump set-up

1. Carry out the perfusion under low light conditions to minimize quenching of fluorescence of the FITC-dextran solution.
2. Assemble all the tools required for perfusion surgery (Figure 1A).
3. Place the perfusion pump at a higher elevation than the rat to be perfused (Figure 1B). This perfusion does not need to be performed in a fume hood since FITC dextran is not hazardous.
4. Fill a 50 mL graduated cylinder with FITC-dextran solution wrapped in foil to block exposure to light and prevent any decrease or quenching of fluorescence. Once you begin perfusion, unwrap the cylinder just enough so you can see the graduation marks on the cylinder.
5. Place the inflow tubing (Tygon, I.D. 1/8, O.D. 1/4, wall 1/16) of the pump into the FITC solution in the beaker and fit the other end of the tubing with a 16 G needle.

6. Start the pump to fill the tubing with FITC-dextran solution. Adjust the pump setting so that the flow rate is 12 mL per minute.
7. Dislodge any air bubbles that form along the lines by flicking the tubing, so that they float to the top and can be eliminated at the other end of the line.
8. Stop the pump immediately when FITC emerges from the 16 G needle. It is important not to have any air bubbles in the lines as they can rupture capillaries and cause false positive extravasation or poor perfusion of the solution.

Perfusion Surgery

1. Inject ketamine (75 mg/kg) and xylazine (5 mg/kg) intraperitoneally into the rat.
2. Pinch the paw to test for absence of paw retraction and ensure that the rat has reached a surgical plane of anesthesia prior to starting the perfusion surgery.
3. Place the rat on a grate over a sink. Turn the water faucet on to help drain the blood that will collect in the sink. The MSDS for FITC-dextran indicates that it is a non-hazardous substance that does not require special disposal procedure.
4. Lift the skin over the ribs using a hemostat, and make a 1 cm cut using a blunt scissors through the abdominal wall under the rib cage.
5. Insert the blunt side of the scissors into the abdominal opening and carefully cut up to the sides of the ribs being careful not to make any cuts or nicks in the liver.
6. Grab the exposed sternum with the hemostat and cut the tissue connecting the liver to the sternum.
7. Make a small incision in the diaphragm and carefully insert the blunt end of the scissors and continue to cut along the entire length to expose the pleural cavity.
8. Using the blunt scissors, cut along one side of the ribs up to the collarbone on both sides, while being careful not to cut or nick the lungs.
9. Lift up the sternum and cut the tissue connecting the heart to the ribs. Make sure that the heart is fully exposed with clear access to the atria and ventricles.
10. Inject heparin (0.35 cc) into the posterior end of the left ventricle and wait 1 min before inserting the 16 G needle, ideally, in the same hole as the heparin injection (Figure 1C).
11. Prior to turning on the pump, make note of how much FITC dextran solution is in the 50 mL graduated cylinder. This will be important as you will want to stop the pump when 12 mL has been used.
12. Turn on the pump and use a small dissecting scissors to make an incision, approximately 5–8 mm, through the right atrium.
13. Make the incision large enough for the blood to leak freely without pulsing.
14. Perfuse 12 mL of FITC-dextran solution at the rate of 12 mL per minute.

15. Turn off the pump once 12 mL of the FITC-dextran has been perfused through the rat (Figure 1D) and remove the needle from the rat's heart.
16. Immediately after perfusion is complete, decapitate the rat using a guillotine.

Brain Removal

1. Remove the skull overlying the brain using a 5.5 inch bone rongeurs (Figure 1E).
2. Carefully use scissors to cut any dura, nerves, or connective tissue
3. Insert a spatula along the side of the skull and tease the brain away from the cranium.
4. Place the brain in a small sample jar filled with 4% PFA and keep at 4°C.

Fixation and Cryoprotection

1. Keep the brain protected from light from this point onwards by wrapping the sample jar in foil and incubate at 4°C for 3 days.
2. After 3 days discard the PFA in the jar and gently rinse the jar and brain with cold 10% glycerol solution.
3. Fill the jar with cold (4°C) 10% glycerol solution and incubate the brain in the 10% glycerol solution for 18–24 hours at 4°C.
4. The following day discard the 10% glycerol solution and rinse the jar and brain with cold 20% glycerol solution.
5. Fill the jar with cold (4°C) 20% glycerol solution and incubate the brain in 20% glycerol solution for 18–24 hours at 4°C.

Flash-Freeze Brains—Brains should then be flash frozen by immersing in cooled 2-methylbutane for 5 min.

Flash-freezing using 2-methylbutane

1. Fill a small 25 mL beaker with 2-methylbutane.
2. Place the beaker in an ice bucket containing a layer of dry ice on the bottom. Surround the beaker with more dry ice.
3. Fill the ice bucket with 95% ethanol so that the dry ice is saturated with ethanol. Avoid introducing dry ice or ethanol into the 2-methylbutane solution and contaminating it.
4. Allow this setup to cool the 2-methylbutane for approximately 3 hrs.
5. Test to see if the 2-methylbutane solution has cooled sufficiently by placing a small piece of brain ($1cm^3$) into the 2-methylbutane solution using a tweezer. If the piece of brain hardens in 5 sec, then the solution is ready for flash freezing a whole brain. This test can be done using a piece of brain tissue that will not be studied in the current experiment.

6. Immerse the brain in 2-methylbutane for at least 1 min but no longer than 5 minutes.

Preparing slides for mounting brain tissue

Subbing slides

1. Place slides in slide holder and soak in tray containing 0.1N hydrochloric acid for 1hr. The number of slides to sub should be based on the experimental design and rostro-caudal extent of the brain region of interest.
2. Remove 0.1N hydrochloric acid and rinse in distilled water 3 times for 30 seconds.
3. Soak in 95% ethanol for 1hr to overnight.
4. Remove ethanol and let slides dry completely.
5. Add gelatin subbing solution to the tray.
6. Immerse slides into the gelatin solution for 2 min.
7. Remove slides and place in 80°C oven for 15 min.
8. Take out slides from oven and allow to cool down for 15 min.
9. Repeat steps 6–8 an additional 3 times.
10. Once slides are completely dry, place them in slide boxes and store in −20°C for long term storage.

Sectioning the brain using the cryostat

1. Maintain the cryostat temperature around −20 to −23°C and keep the cryostat lights off and the room lights dim.
2. Add 1 drop of TissueTek or similar mounting medium to the center of the pedestal and place brain on the TissueTek/mounting medium oriented for the appropriate type section.
3. Add finely powdered dry ice around the brain to freeze the TissueTek/mounting medium.
4. Place pedestal with the brain in the ice bucket and cover brain completely with finely powdered dry ice for 2 min
5. Remove the pedestal containing the brain from the dry ice and apply additional TissuTek/mounting medium around the brain and allow it to set for an additional 2 min.
6. Place the pedestal in the cryostat for at least 2hr to equilibrate with the cryostat chamber temperature before sectioning.
7. Use the cryostat trim setting to advance to the area of interest.
8. Switch from trim to fine setting and set the section thickness to 50 μm and collect the slices.

9. Place a drop of Millipore™ water onto the subbed slide and use a soft bristled paint brush to pick up the tissue section from the blade and place it on the drop of water. The section should unroll or unfold with little effort.
10. Use the paint brush gently and sparingly to help align and orient the section without disrupting or tearing the tissue.
11. Use a Kimwipe® to absorb the water and lay the section flat on the subbed glass slide.
12. Mount up to 3 sections per slide without disrupting previously mounted sections on the slide.
13. Allow sections to air dry completely.
14. Place slides in slide boxes and store at −20°C at this point prior to imaging.

Prepping slides prior to imaging

1. Remove slide from −20°C and immerse in DRAQ5 nuclear stain for 5 min. This step allows for setting the focal plane of the tissue during confocal imaging prior to visualizing fluorescence of FITC- dextran.
2. While the tissue is still wet, apply a line of Fluoromount-G mounting media over each of the 3 tissue sections.
3. Place an appropriate thickness coverslip (based on penetration capability of the objective in the confocal microscope) at one end of the slide and carefully lower it to the other end avoiding the capture of air bubbles.
4. If air bubbles get trapped between cover glass and tissue section, gently press on the cover glass and move the bubble out of the tissue towards the edge.
5. Allow for Fluoromount-G to set overnight at room temperature, in the dark, before imaging.
6. The next day remove any smudges or Fluoromount-G overflow on the slide by wiping with a soft microfiber cloth dipped in 70% ethanol.

Confocal Imaging

1. Use an available confocal microscope to image FITC-dextran extravasation.
2. The necessary parameters for imaging is the availability of 633 nm excitation wavelength laser and an emission collection at 660/20 band pass filter for visualizing DRAQ5 nuclear stain for orienting the focal plane of the tissue.
3. FITC-dextran visualization requires a 488 nm excitation wavelength laser and an emission collection spectrum of 504–556 nm.
4. The images can be captured at 4 μm z steps.
5. Use the same Gain, Offset and exposure settings for all images captured within one experiment.

6. Capture multiple images from the region of interest and from multiple brain sections of the same region.

Image J Software for data analysis

1. Use Image J for analyzing the data obtained from confocal imaging.
2. Download Image J software from https://imagej.nih.gov/ij/
3. Download the Bioformats plugin and install it in the plugins folder as detailed on the website. This plugin is important for opening different types of image formats.

REAGENTS AND SOLUTIONS

- 0.1M and 0.2M Phosphate Buffered Saline (PBS)
  - Sodium chloride
  - Monosodiumphosphate
  - Disodiumphosphate
- 4% paraformaldehyde (PFA) solution
  - 0.2M PBS
  - 6N sodium hydroxide
  - PFA
- 10% and 20% Glycerol solution
  - 0.2M PBS
  - Glycerol
  - Dimethylsulfoxide
  - Millipore™ water
- FITC dextran solution
  - 10,000 Da FITC labeled dextran
  - Cold 0.1M PBS
- Gelatin subbing solution
  - Chromium potassium sulfate
  - 0.1N hydrochloric acid
  - 95% ethanol
  - 70% ethanol
- Brain freezing solution
  - 2-methylbutane

- Dry ice
- 95% ethanol

- Access to Millipore water

0.1M Phosphate Buffered Saline (PBS)

To make 2L of 0.1M PBS:

Sodium chloride (NaCl) – 17.54 g

Monosodiumphosphate (NaH_2PO_4) – 0.36 g

Disodiumphosphate (Na_2HPO_4) – 2.48 g

Dissolve the above reagents in about 1800 mL of Millipore™ water, and then bring up the volume to 2 L using Millipore™ water. Store at 4°C.

0.2M Phosphate Buffered Saline (PBS)

To make 1L of 0.2M PBS:

Sodium chloride (NaCl) – 17.54 g

Monosodiumphosphate (NaH_2PO_4) – 0.36 g

Disodiumphosphate (Na_2HPO_4) – 2.48 g

Dissolve the above ingredients in about 800 mL Millipore™ water, and then bring up the volume to 1L using Millipore™ water. Store at 4°C.

4% Paraformaldehyde (PFA)

To make 2 L of 4% PFA:

1. Heat 1L of Millipore™ water to 95°C and add 80 g of PFA while maintaining temperature between 80–95°C.
2. Stir for 10 min, turn off heat and add a few drops of 6N NaOH until solution turns clear.
3. If the volume of the solution is less than 1L, due to evaporation, add Millipore water to bring the volume to 1L.
4. Add 1L of cold 0.2M PBS to the PFA solution and allow to cool to room temperature.
5. Check pH and adjust to 7.4 and filter solution.
6. Store solution at 4°C for up to 1 month.

10% Glycerol Solution

To make 700 mL solution

0.2M PBS – 350 mL

Glycerol – 70 mL

Dimethylsulfoxide – 14 mL

Millipore™ water – 266 mL

Place a 1 L graduated cylinder on a stir plate and mix together the above solutions using a stir bar. Store at 4°C for up to 1 month.

20% Glycerol Solution

To make 700 mL solution:

0.2M PBS – 350 mL

Glycerol – 140 mL

Dimethylsulfoxide – 14 mL

Millipore™ water – 196 mL

Place a 1 L graduated cylinder on a stir plate and mix together the above solutions using a stir bar until a uniform solution is obtained. Store at 4°C for up to 1 month.

FITC-Dextran Solution

To make 100 mL solution (10 mg/mL):

10,000 Da FITC labeled dextran – 1 g

Cold 0.1M PBS pH 7.4 – 100 mL

Wrap 200 mL beaker in foil and solubilize above ingredients for 30 min to ensure that any clumps that may have formed have completely dissolved. This solution can be made up fresh and/or stored at 4°C for approximately 72 hrs before using it.

Gelatin Subbing Solution

To make 1600 mL subbing solution

Gelatin – 8.23 g

Chromium potassium sulfate – 0.8 g

1. Heat 1600 mL Millipore™ water to 60°C.
2. Add gelatin slowly into the water while stirring to avoid clumping.
3. Once completely dissolved add 0.8g chromium potassium sulfate to gelatin mixture.
4. Filter while hot.
5. Use this solution for subbing slides within 2 hrs of preparation.

COMMENTARY

Dye perfusion is often followed by a saline or PBS perfusion in order to visualize background extravasation in the absence of capillaries. However, the perfusion of the capillaries with PBS after FITC-dextran is not recommended to minimize the chance of

capillary rupture by the perfusion of PBS. Furthermore, image manipulations can be carried out as detailed in the Analysis section, to exclude the fluorescent capillaries without introducing the additional variable of PBS perfusion, which could contribute to false positive results. Additionally, it is possible that the subsequent PBS perfusion can leak out of permeabilized BBB and wash out the FITC-dextran in the surrounding brain parenchyma causing false negative results. Finally, FITC-dextran in the capillaries helps to visualize the density as well as the morphology of capillaries and acts as an indicator of successful perfusion in the animal (Figure 2) whereas the removal of the FITC-dextran precludes validation of the status of perfusion (Figure 3).

Confocal imaging is essential for assessing FITC-dextran extravasation and fluorescence quantification. Confocal imaging allows for collecting specific wavelengths of light emitted by FITC without including scattered light of other wavelengths that may not be specific to FITC. Additionally, the emission spectra can be obtained throughout the depth of the 3-dimensional plane of a tissue section. Thus, the tissue section can be imaged for fluorescence throughout its entire thickness without confounds due to scattered light from one plane affecting fluorescence in another plane within the same tissue.

Post-imaging manipulations to exclude fluorescence in the capillaries removes fluorescence within the capillaries imaged throughout the depth of the tissue and permits the measure of fluorescence in the non-capillary parenchyma only. In this manner, fluorescence from FITC-dextran filled capillaries through the thickness of the tissue section can be excluded (Figure 4). This allows for visualization and quantification of the increased background fluorescence indicative of FITC that has diffused from the leaky capillaries and into the surrounding parenchyma. At the same time, it excludes FITC-dextran confined within capillaries that would otherwise contribute to background fluorescence.

Background Information

Dyes have been used for assessing the integrity of the BBB dating back to the end of the 19th century (Saunders et al., 2014). Dyes such as vital red and brilliant red were initially used to assess BBB permeability, however, these dyes were not very effective in determining damage due to the color of the dye being similar to lysed red blood cells and because the dyes were not stable enough to maintain detectable levels in the blood stream. Azo dyes such as Trypan Blue and Evans Blue were also used as these dyes are relatively stable in the blood due to their ability to bind proteins in the serum and plasma and because lower concentrations of the dyes were sufficient to detect a permeable BBB (Bladin, 2014). Evans Blue has remained a widely used dye to assess BBB damage despite the major drawback that there is free unbound Evans Blue even at low concentrations. Unbound Evans Blue readily diffuses across an intact BBB and is known to bind to brain tissue and can contribute to false positive results or high background (Saunders et al., 2015).

A better alternative to Evans Blue and other molecules for assessing BBB permeability are dextrans labeled with biotin or fluorophores. Dextrans are made up of varying chain lengths of glucose ranging from 4 – 70 kDa. These molecules have high stability and sensitivity and can be utilized for ascertaining the degree of damage to the BBB (Ek et al., 2006; Ek et al., 2001). In addition, since these molecules are not endogenous and cannot be confused with

endogenous molecules, they can be introduced at various times throughout the life cycle of a disease in order to determine the time of BBB disruption and in turn, information regarding causality.

Critical Parameters

1. It is important to use a correct dose of ketamine/xylazine cocktail for anesthesia. The dose should be sufficient for the rat to remain at a surgical plane of anesthesia without being lethal. For good perfusion to occur the heart should be beating at regular intervals when the heparin is injected into the left ventricle. The heparin injection is given in order to prevent platelet aggregation within the blood vessels and to provide a free flow of blood without any back pressure during perfusion, thereby minimizing rupture of small blood vessels and capillaries.
2. The flow of the perfusion solution needs to be maintained at a rate that is not too high as it would increase pressure on small capillaries causing them to rupture. On the other hand pressure has to be maintained sufficiently to perfuse the smallest diameter capillaries.
3. The incision made in the right atrium for blood outflow has to be sufficiently large (~0.5 cm) for the blood to empty freely without building back pressure.
4. The concentration of FITC-dextran solution used in perfusion should be high enough to overcome the dilution by blood in the vessels during perfusion. The optimal concentration of FITC-dextran to use in perfusion should be empirically determined by the experimenter based on the weight of the animal.
5. It is important to ensure that FITC-dextran powder is completely solubilized prior to perfusion and is cold.
6. Rats must be decapitated and the brain removed immediately after perfusion with FITC-dextran is completed. This would minimize artificial leakage of FITC-dextran from the vessels in the absence of active perfusion by the pump.
7. Cryoprotection of the brain is essential for preventing sudden osmotic changes in cells during flash freezing that would burst the cell wall, damage tissue, and contribute to artificial leakage of FITC-dextran.
8. Do not immerse brains in 2-methylbutane for longer than 5 min as it causes fracturing of the tissue.
9. Using subbed slides for mounting tissue sections helps the tissue adhere to and flatten on the glass slide. Additionally, it prevents the sections from lifting off the slide when incubating in DRAQ5.
10. It is essential to equilibrate the brains to the temperature of the cryostat for at least 2 hr. Frequently, the outside of the brain is at a different temperature compared to the inner regions and this interferes with obtaining consistent slice thickness and also affects the quality of the section.

11. The temperature at which the sections are sliced is important. Sectioning when it is too warm will make the tissue clump and stick to itself. In contrast, if the cryostat temperature is too cold, the tissue will crack and roll up tightly and make it difficult to unroll without causing damage to the tissue. Appropriate temperature setting of the cryostat depends on ambient temperature and humidity, and should be determined prior to sectioning of the area of interest by assessing the quality of sections from a region of the brain that is not of interest.

12. After sectioning, the tissue slices should be carefully and gently transferred from the cryostat blade using a thin brush and placed on the slide, as the blood vessels can break due to mechanical agitation and FITC can leak out of the capillaries.

13. Low-light conditions should be maintained when working with the FITC-dextran perfused brain tissue to minimize the degradation of fluorescence due to quenching by ambient bright light. Low ambient light and covering the brains and tissue with foil help maintain fluorescence.

14. Slide mounted tissue sections need to be completely rehydrated in PBS (with or without DRAQ5) prior to Fluoromount-G application and coverslipping. Fluoromount-G is a mounting media that works well on wet tissue but its application to dry dehydrated sections forms numerous small air bubbles during setting of the mounting media.

15. Do not wash the section after incubation with DRAQ5 as it could wash out FITC-dextran and decrease the DRAQ5 signal making it difficult to identify the correct focal plane during imaging.

16. Both control and treated groups need to be imaged in every session. This would take into account any day-to-day variability or unintended changes in the confocal microscope and treatment of the slides.

17. Same brain areas/regions should be imaged in control and experimental groups in one imaging session rather than imaging all the brain regions of one group followed by a different group.

18. Once the imaging parameters are established, the investigator performing the analyses should be blind to the treatment conditions in order to eliminate introduction of bias to imaging measures.

Troubleshooting

1. Too dark or no visible capillaries in a control rat can be due to poor perfusion

    a. Liver clearing can be used as an indicator of perfusion. The liver should begin to clear out in 5 – 10 s after starting perfusion. If this does not happen, readjust the position of the 16 G needle in the ventricle making sure that the beveled tip is not against the wall of the heart or septum.

    b. Increase the volume of FITC-dextran perfused. The amount of FITC-dextran suggested (12 mL) is for rats weighing between 250–300 g.

Heavier rats may require a greater volume of perfused FITC-dextran to fill the small capillaries in the brain.

c. An increase in the pump flow rate could also be tried, however there is a concern that higher speeds and therefore, higher pressure, could cause brain capillaries to rupture and leak FITC-dextran, leading to false positives for BBB disruption. Keep the pump rate consistent for all control and experimental groups so that changes in the rate of the pump do not unintentionally cause variability in BBB disruption.

2. Bright fluorescent speckles all over the section and slides during imaging

   a. Too much disruption of the tissue section when slicing or placing onto subbed slides can rupture blood vessels and capillaries causing FITC-dextran to leak out.

   b. Kimwipes or other lab tissues can leave behind specks and debris that fluoresce at different wavelengths of light and should not be used for cleaning slides. A soft microfiber cloth is a better alternative for wiping slides clean.

   c. The freezing process could also lead to bright fluorescent specks over the tissue. Incubation in the 2-methylbutane for longer than 5 min could cause tissue dehydration and rupture of cell membranes.

Statistical Analyses

Statistical analysis software

1. Any statistical analysis software can be used.

2. The appropriate analysis to be conducted should be determined based on the experimental design for comparisons between treated and untreated groups. For example, in order to determine whether BBB permeability is increased in methamphetamine treated rats compared to saline, a t-test can be carried out. Further details on how to process data from images of FITC-dextran extravasation within the brain is provided below in the “Image Analysis” section.

Image Analysis—Start Image J software and open the raw confocal image file of interest.

Remove Brightly Stained Capillaries

1. Make a projected image using the option on the top menu. Select Image → Stacks → Z project

2. Select Process → Noise → Remove outlier

3. Select Bright Outliers

4. Set Radius and Threshold to remove capillary staining in control image

5. A starting point for adjusting and removing the capillaries can be setting Radius: 20 px and Threshold: 30 px. Adjust these values until the capillaries are fully eliminated in the image (Figure 4B and D).

6. Keep settings the same for all images that will be compared with one another. For all the settings for images from the cortex of different treatment groups of animals should be kept the same; however, the settings for images of the hippocampus can be different from those of the cortex.

Measure the fluorescence of the entire image without capillary staining

1. Select Analyze from the top menu → Measure

2. A box will pop up on the right of the screen with numbers. Copy all the values into an Excel spreadsheet, making note of the rat and image number.

3. Use the "mean" value that is provided to perform calculations.

Example Analysis for Images from Rats Treated with Saline or Methamphetamine

- A total of 4 rats were used for the purpose of this example - 2 rats treated with saline and 2 with methamphetamine. Three images were taken from 3 separate sections of the cortex for each rat using a confocal microscope. Projection images were analyzed using ImageJ and example analysis is presented in Figure 5.
- As mentioned above, the Radius and Threshold were set to 20 px and 30 px, respectively, in all images, to remove bright outliers (Figure 4).
- The remaining fluorescence was measured, as described above, and the mean values for each image were recorded, in the "The ImageJ Mean Measure of Individual Images" column, for example.
- The mean fluorescence of each of the individual images was calculated for each rat, as seen in the "The Mean of the ImageJ Measures" column.
- The mean fluorescence of all Saline treated rats was calculated, and presented in the "Mean of all Saline Treated Rats" column.
- The mean fluorescence of each rat was calculated as a percentage of the Saline treated rats, as indicated in the "Percent of Saline Treated Rats" column.
- The mean and standard error was then calculated for each treatment group, Saline and Methamphetamine, from the "Percent of Saline Treated Rats" values, and are presented in the "Mean Percent of Saline Treated Rats" and "Standard Error" columns, respectively.
- When the number of rats is appropriate for statistical analysis, a t-test could be used to determine whether there is a significant difference in extravagated fluorescence between the 2 treatment groups, Saline and Methamphetamine.

Understanding Results

The following observations are possible:

1. Bright distinct capillaries on a dark background - Distinct fluorescent capillaries filled with FITC-dextran on a dark background indicates intact BBB (Figure 2A).
2. Bright distinct capillaries on a light background - This indicates either that the capillaries are fairly intact but have increased permeability or that the capillaries have ruptured anytime during perfusion, freezing, sectioning, or mounting tissue on slides and are leaking FITC-dextran into the brain parenchyma (Figure 2B). There is a possibility that the capillaries will be also be wider in this case, indicating diffusion of the FITC-dextran outside of the capillary and remaining within the perivascular space.
3. Faint capillaries on a dark background. This is likely due to poor perfusion of the capillaries caused by clots in the blood vessels or rupture of blood vessels during transcardial perfusion (Figure 3).
4. Faint capillaries on a light background could indicate that the capillaries are not intact, have high permeability and could not contain the FITC-dextran, i.e. all of the FITC-dextran has leaked out of the capillaries.

Time Considerations

The preparations of solutions for perfusion, fixation and cryoprotection can take up to 4 hrs. The procedure for subbing slides can take around 5hrs – overnight, including preparation of the subbing solution, slides and for applying multiple layers of gelatin treatment onto the slides. Perfusion, decapitation and whole brain extraction takes approximately 10 min per rat. Once the brain is extracted, it is placed in 4% PFA at 4°C for 3 days, followed by serial overnight immersion in 10% and 20% glycerol for cryoprotection. Cooling down the 2-methylbutane solution for freezing the brain can take between 2–4 hrs and freezing the brain takes 5 min per brain. Mounting and equilibrating the brain to cryostat temperature take approximately 3 hrs. The time taken for sectioning each brain depends on the region/s of interest and can take several hours. Once the sections are placed and spread with a drop of water on the slides, air drying the section to help adhere them to the slides takes about 30 min. DRAQ5 nuclei staining of slides takes approximately 10 min and coverslips are set overnight after applying the mounting media on the sections. Imaging the tissue sections can take anywhere from several hours to days depending on the region/s of interest and number of slides to image. Once the images are captured, data manipulation and analysis takes around 15 min per image.

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Significance Statement

Increased permeability of the blood-brain barrier (BBB) can often lead to detrimental consequences, including neuropathology, when harmful agents that normally do not cross into the brain from the circulatory system can access the brain. On the other hand, it may be desirable to increase the permeability of the BBB to deliver therapeutic agents that normally cannot cross the BBB. Hence, a reliable method is needed to assess the integrity of the BBB. The peripheral perfusion of a large fluorescently labeled molecule that does not normally penetrate the BBB, such as fluorescein isothiocyanate (FITC) labeled dextran, and its measurement in the brain would be a sensitive indicator of a possible disruption of the BBB.

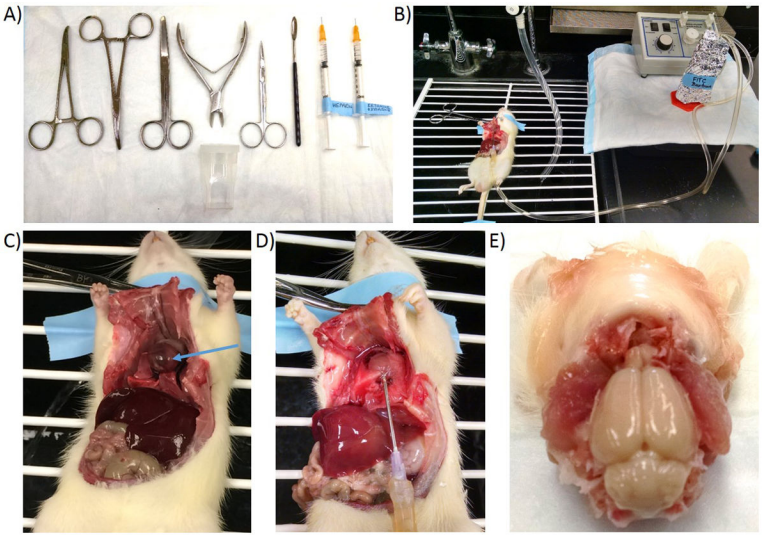

Figure 1.

Perfusion setup and surgery. **A)** Tools required for perfusion. **B)** Perfusion setup showing FITC-dextran solution in a graduated cylinder wrapped in foil. The inflow tube of the peristaltic perfusion pump is placed in the FITC-dextran solution and the outflow tube is attached to a 19 G needle that is inserted into the left ventricle. **C)** Image of a rat ready for perfusion with FITC-dextran. Arrow denotes the location where the needle will be inserted in the left ventricle. Note the dark coloration of the liver prior to starting perfusion. **D)** Image of a rat after perfusion. Note the lighter color of the liver. **E)** Image of brain prior to removal from the cranium. Note the absence of pink coloration of the brain due to perfusion of the blood vessels.

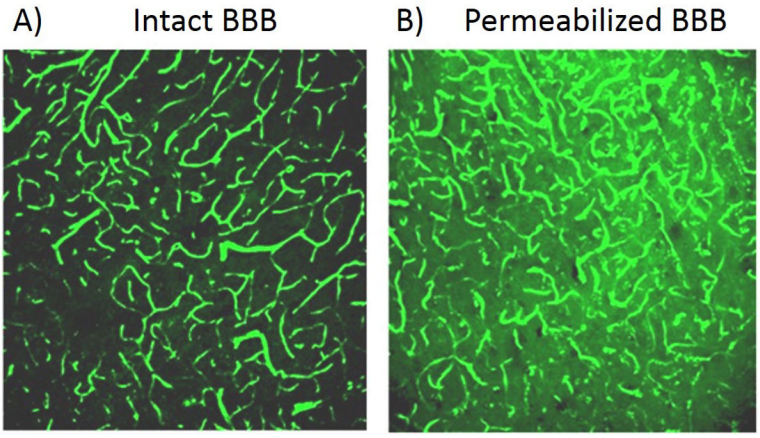

Figure 2.

Cortical capillaries after FITC-dextran perfusion. **A)** FITC-dextran extravasation in a control animal. Image shows capillaries filled with brightly fluorescent FITC-dextran on a dark background indicative of uncompromised BBB. **B)** Cortical capillaries after FITC-dextran perfusion in a rat treated with methamphetamine demonstrating a permeable BBB. Image shows capillaries filled with brightly fluorescent FITC-dextran on a bright green background indicative of extravasation of FITC-dextran beyond the BBB.

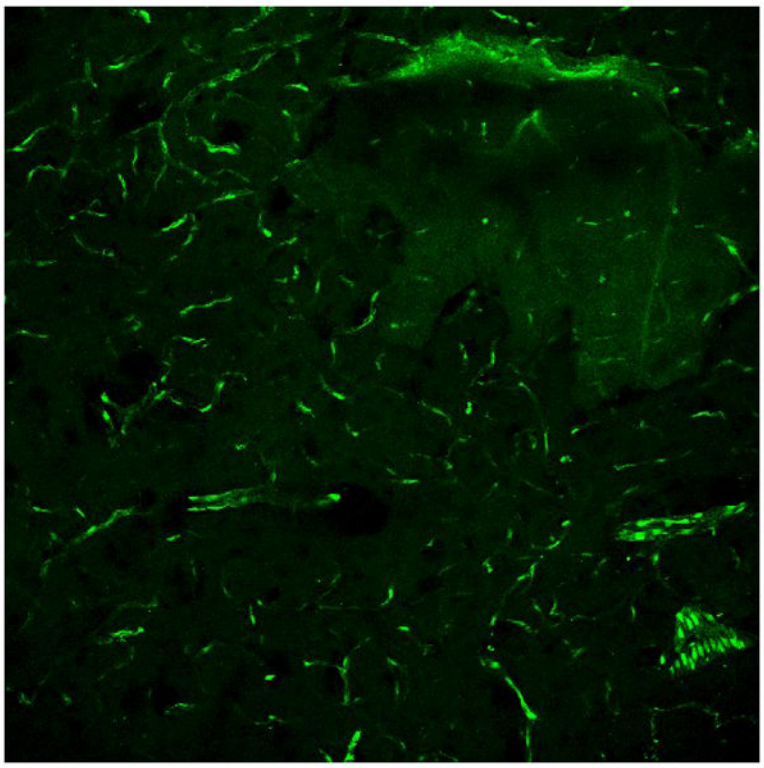

Figure 3.
Poor FITC-dextran perfusion of cortical capillaries. Image shows capillaries that are sparsely filled with FITC-dextran.

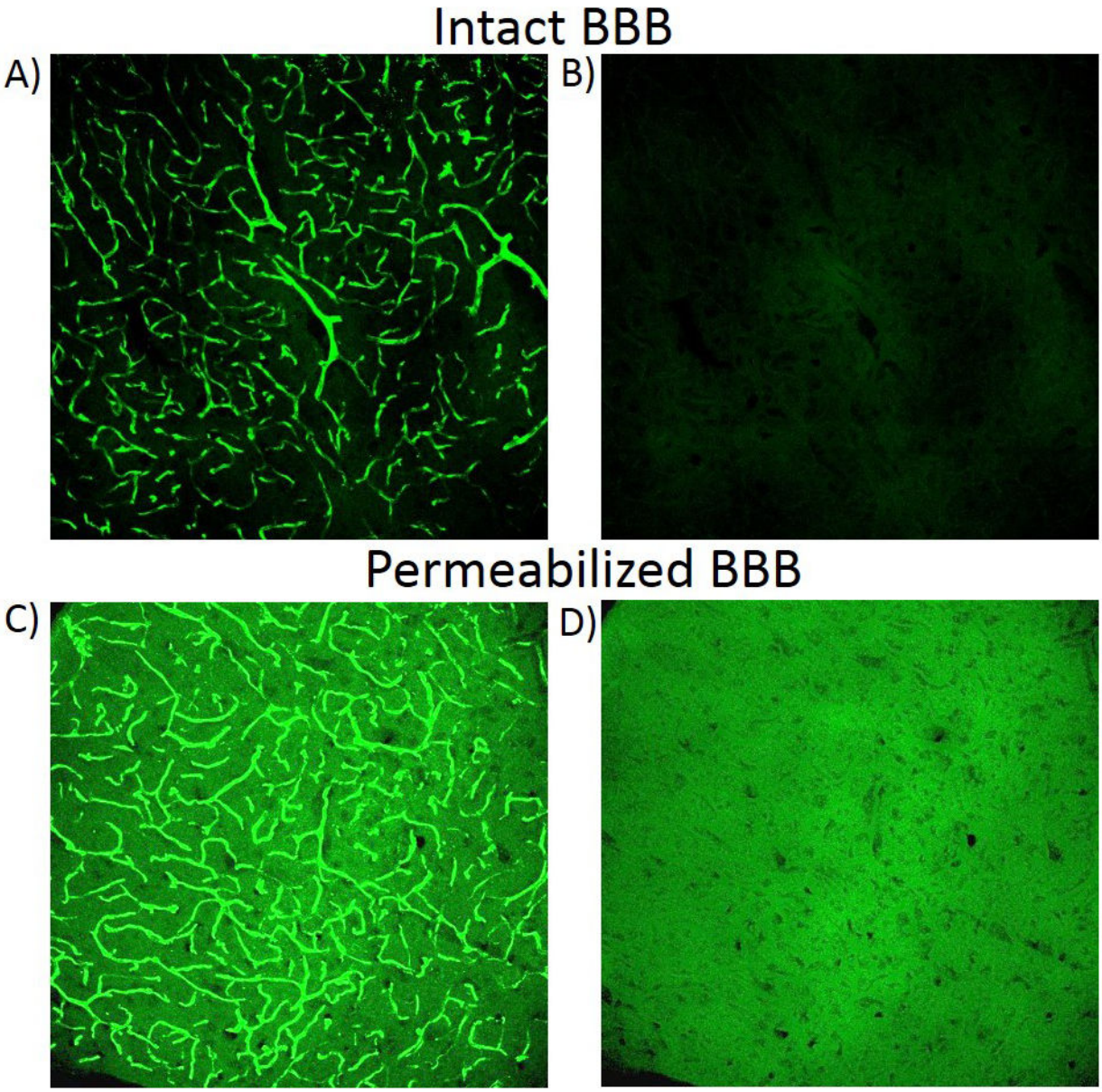

Figure 4.

Image manipulation to remove cortical capillaries after FITC-dextran perfusion. **A**) FITC-dextran extravasation in an animal with uncompromised capillaries showing brightly fluorescent FITC dextran filled capillaries on dark background. **B)** Cortical capillary fluorescence removed from the image presented in panel A using Image J software to visualize FITC dextran extravasation into the brain parenchyma in a BBB intact rat. **C)** FITC-dextran extravasation in an animal with compromised capillaries showing brightly fluorescent FITC-dextran filled capillaries on a bright background. **D)** Cortical capillary fluorescence removed from the image presented in panel C using Image J software to visualize FITC-dextran extravasation into the brain parenchyma in a rat with a permeabilized BBB.

	The ImageJ Mean Measure of Individual Images	The Mean of the ImageJ Measures
Saline 1		
image 1	2.518	4.341
image 2	2.769	
image 3	5.901	
image 4	5.632	
image 5	4.729	
image 6	5.445	
image 7	3.458	
image 8	4.629	
image 9	3.984	
Saline 2		
image 1	3.663	3.344
image 2	3.138	
image 3	3.807	
image 4	3.195	
image 5	3.323	
image 6	2.111	
image 7	4.521	
image 8	3.211	
image 9	3.128	
Methamphetamine 1		
image 1	7.215	8.579
image 2	10.76	
image 3	9.917	
image 4	6.055	
image 5	12.592	
image 6	6.033	
image 7	7.983	
image 8	8.598	
image 9	8.054	
Methamphetamine 2		
image 1	5.972	6.714
image 2	5.62	
image 3	7.99	
image 4	6.719	
image 5	5.768	
image 6	7.703	
image 7	6.931	
image 8	7.629	
image 9	6.097	

	The Mean of the ImageJ Measures	Mean of all Saline Treated Rats	Percent of Saline Treated Rats
Saline 1	4.341	3.842	112.967
Saline 2	3.344		87.033
Methamphetamine 1	8.579		223.264
Methamphetamine 2	6.714		174.746

	Mean Percent of Saline Treated Rats	Standard Error
Saline (n=2)	100.000	12.967
Methamphetamine (n=2)	199.005	24.259

Figure 5.

Example analysis of images from Saline and Methamphetamine treated rats. For the purpose of this example, a total of 4 rats were used, 2 saline treated and 2 methamphetamine treated. Three images were taken from 3 separate brain sections of the cortex. The individual mean fluorescent values for each image, after removal of the bright outliers, are listed for each image for each rat. Additional values are also presented, indicating the calculations made to arrive at the final presentation of the data, which was the average fluorescence for each treatment group as a percent of the controls (Saline).