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## Visualizing impairment of the endothelial and glial barriers of the neurovascular unit during experimental autoimmune encephalomyelitis in vivo --Manuscript Draft--

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**TITLE:**

**Visualizing Impairment of the Endothelial and Glial Barriers of the Neurovascular Unit During Experimental Autoimmune Encephalomyelitis In vivo**

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**KEYWORDS:**

experimental autoimmune encephalomyelitis, in vivo permeability, in situ zymography, blood-brain barrier, glia limitans, neurovascular unit

**SUMMARY:**

Here, we present protocols to investigate impairment of the neurovascular unit during experimental autoimmune encephalomyelitis in vivo. We specifically address how to determine blood-brain barrier permeability and gelatinase activity involved in leukocyte migration across the glia limitans.

**ABSTRACT:**

The neurovascular unit (NVU) is composed of microvascular endothelial cells forming the blood-brain barrier (BBB), an endothelial basement membrane with embedded pericytes, and the glia limitans composed by the parenchymal basement membrane and astrocytic end-feet embracing the abluminal aspect of central nervous system (CNS) microvessels. In addition to maintaining CNS homeostasis, the NVU controls immune cell trafficking into the CNS. During immune surveillance of the CNS, low numbers of activated lymphocytes can cross the endothelial barrier without causing BBB dysfunction or clinical disease. In contrast, during neuroinflammation, such as in multiple sclerosis or its animal model, experimental autoimmune encephalomyelitis (EAE), a large number of immune cells can cross the BBB and subsequently the glia limitans, eventually reaching the CNS parenchyma leading to clinical disease.

Immune cell migration into the CNS parenchyma is thus a two-step process that involves a sequential migration across the endothelial and glial barrier of the NVU employing distinct molecular mechanisms. If, following their passage across the endothelial barrier, T cells encounter their cognate antigen on perivascular antigen-presenting cells, their local reactivation

will initiate subsequent mechanisms leading to the focal activation of gelatinases, which will enable the T cells to cross the glial barrier and enter the CNS parenchyma. Thus, assessing both BBB permeability and MMP activity in spatial correlation to immune cell accumulation in the CNS during EAE allows specification of loss of integrity of the endothelial and glial barriers of the NVU. We show here how to induce EAE in C57BL/6 mice by active immunization and subsequently analyze BBB permeability in vivo using a combination of exogenous fluorescent tracers. We further show how to visualize and localize gelatinase activity at the glia limitans in EAE brains by in situ zymography combined with immunofluorescent staining of brain barriers basement membranes and CD45<sup>+</sup> invading immune cells.

## INTRODUCTION:

The central nervous system (CNS) coordinates all body and mental functions in vertebrates, and CNS homeostasis is essential for a proper communication of neurons. CNS homeostasis is warranted by the neurovascular unit (NVU), which protects the CNS from the changing milieu of the blood stream. The NVU is composed of CNS microvascular endothelial cells, which are biochemically unique and establish the blood-brain barrier (BBB) in continuous crosstalk with pericytes, astrocytes, neurons, and extracellular matrix (ECM) components, establishing two distinct basement membranes<sup>1</sup>. The endothelial basement membrane that ensheathes the abluminal aspect of the BBB endothelial cells harbors a high number of pericytes and is composed of laminin  $\alpha$ 4 and laminin  $\alpha$ 5, in addition to other ECM proteins<sup>2</sup>. In contrast, the parenchymal basement membrane consists of laminin  $\alpha$ 1 and laminin  $\alpha$ 2 and is embraced by astrocytic end-feet. The parenchymal basement membrane together with the astrocyte end-feet composes the glia limitans that segregates the CNS neuronal network from the cerebrospinal fluid filled perivascular or subarachnoid spaces<sup>3</sup>. Due to the unique architecture of the NVU, immune cell trafficking into the CNS is distinct from that into peripheral tissues as it requires a two-step process with the immune cells, first breaching the endothelial BBB and subsequently the glia limitans in order to reach the CNS parenchyma.

Multiple sclerosis (MS) is a common neuroinflammatory disease of the CNS, in which a large number of circulating immune cells enter the CNS and cause neuroinflammation, demyelination, and focal loss of BBB integrity<sup>4</sup>. Loss of BBB integrity is an early hallmark of MS, as indicated by the presence of gadolinium contrast enhancing lesions in the CNS as visualized by magnetic resonance imaging (MRI)<sup>5</sup>. Leukocyte extravasation into the CNS occurs at the level of postcapillary venules; however, the precise mechanisms involved in immune cell diapedesis across the BBB basement membrane and subsequently the glial barrier remain to be explored. Experimental autoimmune encephalomyelitis (EAE) serves as an animal model for MS and has significantly contributed to our current knowledge about MS pathogenesis. For instance, using the EAE model it has been discovered that leukocyte extravasation occurs in a multistep process, including an initial capture and rolling step mediated by selectins and mucin-like molecules such as P-selectin glycoprotein ligand (PSGL)-1, followed by integrin-dependent firm arrest and crawling of T cells on BBB endothelial cells to permissive sides for diapedesis<sup>6</sup>.

Once T cells have crossed the endothelial BBB and the endothelial basement membrane, they

need to encounter their cognate antigen on macrophages or dendritic cells strategically localized in the leptomeningeal or perivascular spaces. This interaction induces focal production of pro-inflammatory mediators that trigger the subsequent mechanisms required for CNS tissue invasion of immune cells via the glia limitans<sup>7-9</sup>. Focal activation of matrix-metalloproteinases (MMP) -2 and MMP-9 alters chemokine activation and induces degradation of extracellular matrix receptors on astrocyte end-feet, which is a prerequisite for immune cell migration across the glia limitans into the CNS parenchyma and to induce the onset of clinical symptoms of EAE<sup>10, 11</sup>.

Combining detection of CNS infiltrating immune cell with BBB leakage and gelatinase activity in CNS tissue sections provides valuable information about the functional integrity of the endothelial and glial barrier in the context of neuroinflammation. For instance, we recently investigated the constitutive loss of the endothelial tight junction molecule junctional adhesion molecule (JAM)-B in immune cell trafficking into the CNS in the context of EAE. Compared to healthy wild-type C57BL/6 mice, healthy JAM-B-deficient littermates showed no impairment of BBB integrity as shown by *in vivo* permeability assessment using endogenous as well as exogenous tracers<sup>12</sup>. In the context of EAE, JAM-B-deficient C57BL/6 mice showed ameliorated disease symptoms, which was associated with inflammatory cell trapping in the leptomeningeal and perivascular spaces<sup>12</sup>. To examine this phenomenon we applied *in situ* zymography, allowing identification of gelatinase activity in order to test if lack of gelatinase activity in JAM-B-deficient mice may be responsible for the reduced numbers of immune cells able to breach the glia limitans<sup>12</sup>.

Given the availability of different genetically modified mouse models lacking, e.g., different BBB tight junction molecules that might cause changes in BBB function, methodologies for investigating BBB integrity are important. In addition, newly developed drugs could impact on NVU barriers. Here we show how to induce EAE in C57BL/6 mice by active immunization with the myelin oligodendrocyte glycoprotein (MOG)-peptide aa35-55 in complete Freund's adjuvants. We then explain how to localize immune cell infiltration across the endothelial and glial barriers of the NVU and how to study *in vivo* endothelial and glial barrier integrity by *in situ* detection of exogenous tracers and gelatinase activity, respectively.

## **PROTOCOL:**

All studies were conducted under the guidelines according to the Swiss legislation on the protection of animals and were approved by the Veterinary Office of the Canton of Bern, Switzerland (permission numbers: BE 31/17 and BE 77/18).

### **1. Housing of C57BL/6 mice in specific pathogen free conditions**

1.1. House mice in individual ventilated cages with a 12/12 h light-dark cycle. Provide food and water *ad libitum*.

1.2. Use ear punches to individually mark 8-12 week old female C57BL/6 mice.

## 2. Immunization of C57BL/6 mice

### 2.1. Preparation of solutions<sup>13</sup>:

2.1.1. For Complete Freund's Adjuvant (CFA), mix 30 mL of Incomplete Freund's Adjuvant with 120 mg of freshly pestled heat inactivated Mycobacterium tuberculosis (H37RA) under a fume hood. Store stock solution at 4 °C.

2.1.2. For the MOG<sub>aa35-55</sub>-peptide stock solution, dissolve MOG<sub>aa35-55</sub>-peptide in sterile PBS (4 mg/mL) and store at -80 °C.

### 2.1.3. Prepare the MOG-emulsion:

2.1.3.1. Dilute CFA 1:2 with MOG-peptide stock solution in a 2 mL microtube.

2.1.3.2. Seal the microtube with sealing film and fix on a Vortex mixer by completely covering the tube with adhesive tape. Vortex emulsion for 4 h at 4 °C.

### 2.1.4. Prepare syringes with stabile emulsion:

2.1.4.1. Take the microtube and quickly spin down the emulsion using a small table centrifuge. Collect emulsion into a syringe using a 18G x 1½" (1.2 mm x 40 mm) injection needle.

2.1.4.2. According to the number of mice adjust the emulsion volume in the syringe (100 µL/mouse) and replace the 18G needle with a 27G x ¾" (0.4 mm x 19 mm) injection needle. Seal the injection needle attached to the syringe with sealing film.

2.1.5. For the pertussis toxin (PTx) stock solution, dissolve 50 µg of lyophilized PTx in 500 µL of sterile PBS (100 ng/µL). Store stock solution at 4 °C.

2.1.6. For the PTx working solution, mix 97 µL of sterile PBS with 3 µL of PTx stock solution (300 ng/100 µL) per mouse (for intraperitoneal injection use a 30G x ½" (0.3 mm x 13 mm) needle).

## 2.2. EAE induction

2.2.1. Anesthetize C57BL/6 mice with isoflurane using a vaporizer system. To induce anesthesia in mice, expose the mouse to 4.5% isoflurane in oxygen in a small incubation chamber before transferring the mouse to a facemask with 2.1% isoflurane.

2.2.2. Fix the anesthetized mouse in one hand and first inject 30 µL of MOG<sub>35-55</sub>/CFA-emulsion subcutaneously into hind leg flanks (**Figure 1: 1 + 2**; in total 60 µL; left flank 30 µL and right flank 30 µL) in close proximity to the inguinal lymph nodes.

2.2.3. Place the mouse on its belly and inject 20  $\mu$ L of MOG<sub>aa35-55</sub>/CFA-emulsion into the left and right soft fatty tissue at the tail root (**Figure 1**: 3 + 4; in total 40  $\mu$ L; left side tail root 20  $\mu$ L and right side tail root 20  $\mu$ L) and a little droplet of MOG<sub>aa35-55</sub>/CFA-emulsion into the neck of the mouse (**Figure 1**: 5).

2.2.4. Inject 100  $\mu$ L of PTx solution intraperitoneally into the mouse. Hold the head of the mouse below the body center avoiding injection into the intestine.

2.2.5. Replace maintenance diet (extrudate major nutrients: crude protein 18.5%; crude fat 4.5%; crude fiber 4.5%; crude ash 6.5%; starch 35%; metabolic energy: 13.1 MJ/kg) to breeding diet (extrudate major nutrients: crude protein 23.5%; crude fat 5.5%; crude fiber 3%; crude ash 5.7%; starch 36%; metabolic energy: 14.3 MJ/kg) in order to provide the mice with food of higher energy content before and during the expected clinical disease.

2.2.6. Remove the face mask and make sure the mouse is fully awake and motile after 10 minutes.

2.2.7. Repeat the PTx injection (2.2.4) 48 h after the first treatment.

### **3. Scoring of EAE mice**

3.1. Check the health status of EAE mice every morning by taking a look inside the cages.

3.2. Score EAE mice every afternoon.

3.3. Take every individual mouse included in the EAE experiment out of the cage and check whether the tail has tonus by moving it upward with a finger. A healthy mouse will keep its tail up (the tail has a tonus). If clinical EAE has started, the tail tonus will be lower, visible by a gradual drop of the tail. Eventually the mouse will not be able to lift its tail at all.

3.4. Place every individual mouse included in the EAE experiment on the clean bench and observe and document the walking behavior. See **Table 1**<sup>14</sup> for scoring criteria for the assessment of disease severity (the EAE score).

3.5. Assess and document the weight of every mouse included in the experiment.

### **4. In vivo permeability assay**

#### **4.1. Preparations of solutions:**

4.1.1. For dextran stock solutions, dissolve 10 mg of 10 kDa Dextran Alexa Fluor 488 as well as 3 kDa Dextran Texas Red in 500  $\mu$ L of 0.9% sodium chloride solution (20 mg/mL).

4.1.2. For the dextran working solution, just before injection pipet 55  $\mu$ L of 10 kDa Dextran Alexa Fluor 488 stock solution (20 mg/mL) onto a piece of sealing film and add 55  $\mu$ L of 3 kDa dextran Texas Red stock solution (20 mg/mL). Mix and collect 100  $\mu$ L into a disposable fine syringe (final concentration 2 mg/100  $\mu$ L).

4.1.3. For the 10% formaldehyde (PFA) stock solution, combine 10 g of PFA extra pure powder, 100 mL of PBS, and 200  $\mu$ L of 1N NaOH in a clean glass beaker and heat up to precisely 56 °C under stirring using a magnetic stirrer; keep at 56 °C for 30 min until the PFA is completely dissolved; cool down to room temperature; adjust pH to 7.4; and filter through a paper filter. Store at - 20 °C. The stock solution can be diluted further using PBS.

4.2. Shortly anesthetize healthy C57BL/6 mice or C57BL/6 mice suffering from EAE with isoflurane (the mouse will be sedated for approximately 1 min). Use an automated system as in step 2.2.1 (mice will be exposed to 4.5% isoflurane in oxygen in an induction chamber and then transferred to a facemask with 2.1% isoflurane).

4.3. Place the anesthetized mouse in lateral position on the table, then intravenously (e.g. retro-orbitally) inject 100  $\mu$ L of fluorescent tracers into the mouse before the mouse wakes up.

4.4. Immediately remove the facemask and make sure the mouse is fully awake and motile after the short anesthesia. Let the tracer circulate for 15 min.

4.5. Proceed with step 5.1.

## 5. Perfusion of mice

### 5.1. For *in vivo* permeability assessment:

5.1.1. Begin the procedure 15 min after fluorescent tracer injection by inducing deep isoflurane anesthesia. Use the paw withdrawal reflex to judge the depth of the anesthesia (pinch the skin between the toes; a lack of flexion indicates a sufficient depth), and probe the reflexes of both the forelimb and the hindlimb in mice subjected to EAE.

5.1.2. Fix the mouse on its back and spray the belly with ethanol. Open the thorax using a scissor and remove the diaphragm. Open the right atrium of the beating heart using a scissor and perfuse the mouse through the left ventricle of the heart with 10 mL of PBS followed by 10 mL of 4% PFA in PBS.

CAUTION: To avoid inhaling PFA, perfuse the mouse in a fume hood.

5.1.3. Proceed to section 6.

### 5.2. For *in situ* zymography:

5.2.1. Induce deep isoflurane anesthesia in a mouse suffering from EAE, and check the depth of anesthesia as in step 5.1.1.

5.2.2. Next, fix the mouse on its back and spray the belly with ethanol. Open the thorax using a scissor and remove the diaphragm. Open the right atrium of the beating heart using a scissor and perfuse the mouse through the left ventricle of the heart with 20 mL of PBS.

5.2.3. Proceed to section 6.

## **6. Dissection and freezing of brains**

### **6.1. Preparation of cooling bath:**

6.1.1. Fill a flat dewar container with crushed dry ice and add 2-methylbutane (Isopentane) until the liquid reaches about 1 cm above the dry ice pack. Cover with a loose lid – e.g., an ice bucket cover.

6.2. Clip off the head of the perfused mouse with sharp scissors and remove skin and ears using a smaller set of scissors.

6.3. Carefully dissect the skullcap by cutting from the foramen magnum towards the front at the left and the right side allowing to upfold the skullcap over the brain. Then, carefully lift out the intact brain from the base of the skull while severing the optical nerves using a flat metal spatula.

6.4. Place brain on aluminum foil and cut the brain in three pieces (frontal brain, middle brain, and cerebellum + brain stem) by placing two coronal cuts.

6.5. Fill a cryomold (25 mm x 20 mm x 5 mm) to the first quarter with optimum temperature cutting (O.C.T.) matrix, place brain slices with the anterior side of each brain piece facing downwards into the cryomold, and cover tissue completely with O.C.T. matrix.

6.6. Place the cryomold with the tissue in a horizontal orientation into the 2-Methylbutane bath and make sure that the tissue freezes from the bottom to the top within 1 minute by avoiding dipping the entire tissue block into the bath.

6.7. Transfer frozen tissue to -80 °C for storage and proceed with section 7.

## **7. Preparation of frozen tissue sections**

7.1. Equilibrate temperature from frozen tissue from -80 °C to -20 °C in the cryostat for 30 min.

7.2. Remove tissue block from the cryomold and mount on the cryostat tissue holder (set to -15 °C) using O.C.T. matrix.



7.3. Trim the edges of the tissue block on the holder of the cryostat with a sharp scalpel, and start cutting into the tissue block by removing 20 µm sections with the cryostat knife until the tissue is visible.

#### 7.4. For the analysis of *in vivo* BBB permeability:

7.4.1. Cut 6-10 µm tissue sections, collect them on adhesion glass slides, and immediately image the sections covered with a cover slip using a fluorescence microscope.

7.4.2. Assess appearance of blood vessels in the brain (pay attention to sharp borders of non-leaky blood vessels compared to diffuse fluorescence patterns observed for leaky blood vessels). As positive control, verify the leakage of the tracer into the stroma of the circumventricular organs or the choroid plexus which lack a BBB.

#### 7.5. For *in situ* zymography:

7.5.1. Cut 6 µm tissue sections using a cryostat, collect them on adhesion glass slides, and freeze tissue sections at -20 °C in a freezing box containing silica gel in the lid.

### 8. *In situ* zymography combined with laminin/CD45 immunofluorescence staining

#### 8.1. Prepare solutions:

##### 8.1.1. Prepare embedding solution:

8.1.1.1. Mix 6 g of glycerol (analytical grade), 2.4 g of poly(vinyl alcohol), 6 mL of ddH<sub>2</sub>O, and 12 mL of 0.2 M Tris buffer, pH 8, and stir (magnetic stirrer) the solution for 4 h at RT.

8.1.1.2. Transfer the solution to a 50 mL centrifuge tube and let it rest for 2 h at RT. Subsequently incubate the solution for 10 min at 50 °C (water bath) and centrifuge for 20 min at 2,700 x *g* at room temperature.

8.1.1.3. Take the supernatant and freeze in aliquots at -20 °C.

##### 8.1.2. Prepare cold gelatin solution:

8.1.2.1. Dissolve 0.1 g of cold gelatin from bovine skin in 10 mL of ddH<sub>2</sub>O.

8.1.2.2. Let it soak for at least 1 day and store aliquots at 4 °C.

##### 8.1.3. Prepare ice-cold methanol (-20 °C):

8.1.3.1. Put 100% methanol in a coplin jar and cool down to -20 °C.

8.1.4. Prepare 10% sodium azide stock solution:

8.1.4.1. Add 1 g of sodium azide to 10 mL of ddH<sub>2</sub>O and store at room temperature.

8.1.5. Prepare 0.1% sodium azide working solution:

8.1.5.1. Mix 99 µL of ddH<sub>2</sub>O with 1 µL of 10% sodium azide stock solution.

8.1.6. Dissolve 1 mg of fluorescein conjugated dye-quenched (DQ) gelatin from pig skin in 1 mL of sodium azide working solution and store 100 µL aliquots protected from light at 4 °C.

8.1.7. Dissolve 30 mg of 1,10-phenanthroline monohydrate in 76 µL of ethanol and store at -20 °C.

8.1.8. Prepare 25x protease inhibitor solution:

8.1.8.1. Add 1 tablet of EDTA free protease inhibitor to 2 mL of ddH<sub>2</sub>O. Store at -20 °C.

8.1.9. Just before use, prepare reaction solution (150 µL/section):

8.1.9.1. Centrifuge the 1 mg/mL DQ gelatin solution for 5 min at 13,400 x *g*.

8.1.9.2. Mix 127.2 µL of ddH<sub>2</sub>O, 15 µL of 10x reaction buffer (Gelatinase/Collagenase Assay Kit; see **Table of Materials**), 0.3 µL of 20 mg/mL cold gelatin, 1.5 µL of 1 mg/mL DQ gelatin, and 6 µL of 25x protease inhibitor solution.

8.1.10. Just before use, prepare phenantroline negative control reaction solution (150 µL/section):

8.1.10.1. Mix 125.2 µL of ddH<sub>2</sub>O, 15 µL of 10x reaction buffer, 0.3 µL of 20 mg/mL cold gelatin, 1.5 µL of 1 mg/mL DQ gelatin, 6 µL of 25x protease inhibitor EDTA free, and 2 µL of 2 M 1,10-phenantroline (MMP inhibitor).

8.1.11. Just before use, prepare cold gelatin (non-fluorescent) negative control reaction solution (150 µL/section):

8.1.11.1. Mix 128.7 µL of ddH<sub>2</sub>O, 15 µL of 10x reaction buffer, 0.3 µL of 20 mg/mL cold gelatin, and 6 µL of EDTA-free 25x protease inhibitor.

8.1.12. Prepare primary antibody cocktail for counterstaining, for example, 0.95 µg/mL polyclonal rabbit anti-laminin antibody and 10 µg/mL polyclonal rat anti-CD45 antibody in 1% BSA in PBS, and prepare appropriate isotype control primary antibody cocktails.

8.1.12.1. Prepare secondary antibody solution, for example, 7.5 µg/mL Cy3 goat anti-rat + 15 µg/mL AMCA anti-rabbit in 1% BSA in PBS (protect from light).

8.2. Thaw 6 µm non-fixed brain tissue sections from EAE mice inside the plastic freezing box containing silica gel in the lid in a fume hood to avoid retention of water to the tissue and separate 2 tissue sections on one slide by drawing lines with a water repelling pen

8.3. Prepare reaction solutions (steps 8.1.9-8.1.11), centrifuge for 5 min at 13,400 x g at room temperature, and pre-warm to 37 °C using a water bath.

8.4. Rehydrate the tissue sections for 5 min at 37 °C using 1x reaction buffer. Then, pour off the 1x reaction buffer, add reaction solution on the tissue sections, and incubate for 4 h at 37 °C. Wash slides 5 times in ddH<sub>2</sub>O.

8.5. Fix sections for 5 min with ice-cold methanol at -20 °C and afterwards wash sections once with PBS at room temperature.

8.6. Add 1% BSA in PBS to the sections and incubate for 20 min at room temperature (protect from light). Then, discard 1% BSA in PBS from the sections by flipping the slides on tissue, add primary antibody cocktail, and incubate for 1 h at room temperature (protect from light).

8.7. Wash sections twice with PBS, add secondary antibody cocktail, and incubate for 1 h at room temperature (protect from light).

8.8. Wash sections twice with PBS, mount the slides with embedding solution, let mounted sections dry over night at room temperature (protect from light). Finally, analyze stained tissue sections using a fluorescent microscope.

## REPRESENTATIVE RESULTS:

Assessment of the clinical course of EAE in C57BL/6 mice should result in a disease curve as depicted in **Figure 2A** and changes in the mouse body weight as presented in **Figure 2B**. C57BL/6 mice immunized with MOG<sub>aa35-55</sub> usually start to develop disease symptoms around day 10-12 after active immunization (**Figure 1A**). Typically, immunized mice show a transient drop of body weight the day after the injection of the emulsion and the first dose of pertussis toxin (**Figure 1B**). From day 2 after EAE induction, the body weight of immunized mice then gradually increases until the disease starts, when the body weight typically drops one day prior to the development of clinical symptoms and continues to decrease correlating to the increasing disease symptoms (**Figure 1A,B**). The EAE disease severity increases until the peak of the disease, which can be expected between days 16 and 20 after EAE induction (**Figure 2A**). At the peak of clinical EAE, mice also show the lowest body weight (**Figure 2B**), which increases in correlation to amelioration of EAE in the remission phase of the disease (**Figure 2A,B**). EAE induction in C57BL/6 mice using MOG<sub>aa35-55</sub> results in a chronic disease pathology, and mice typically do not fully recover (**Figure 1A**).

**Figure 3A** shows a representative picture of the choroid plexus and the adjacent brain parenchyma of a C57BL/6 mouse suffering from EAE that was intravenously injected with fluorescent 3 kDa Dextran (red) and 10 kDa Dextran (green) 15 min before sacrificing; scale bar 50  $\mu$ m. As we have previously demonstrated, tracers readily diffuse across the fenestrated microvessels into the stoma of the choroid plexus (left and middle picture), while the BBB does not allow the diffusion of circulating tracers into the brain parenchyma, indicated by the dashed line (right picture), which is thus devoid of any fluorescent signal. **Figure 3B** shows representative leaky blood vessels in the cerebellum of a C57BL/6 mouse suffering from EAE that was intravenously injected with fluorescent 3 kDa Dextran (red) and 10 kDa Dextran (green) 15 min before sacrificing. Arrow heads indicate tracers diffused across the brain microvessels into the CNS parenchyma (left and middle picture), suggesting that the BBB function is impaired in these vessels. In the CNS parenchyma, green and red fluorescent signal is visible outside of the blood vessel walls, which are indicated by dashed lines. In EAE, in the cerebellum where inflammatory cuffs appear the tracers can also be found in the perivascular space between the endothelial basement membrane and the glia limitans indicating functional loss of endothelial BBB integrity. If tracer is found to diffuse into the brain parenchyma, it shows additional dysfunction of the glial barrier<sup>12</sup>.

**Figure 4** shows representative pictures of the analysis of gelatinase (MMP) activity in an EAE brain of a C57BL/6 mouse visualized by in situ zymography and localized to specific brain barrier compartments by anti-pan-laminin (blue) and to the presence of inflammatory cells by anti-CD45 (red) immunofluorescence staining. Proteolytic cleavage of dye-quenched (DQ) gelatin unquenches a fluorescein signal, which allows localization of gelatinase activity on the tissue section. When cold gelatin (non-fluorescent control) is incubated, no green fluorescent signal can be detected in the brain sections from the EAE mice (upper row) as well as in sections that have been incubated with DQ-gelatin combined with phenantroline, a potent Zn<sup>+</sup>-complexing agent and MMP inhibitor (middle row). In the lower row, focal gelatinase/MMP activity is visible as typical granular bright green fluorescence signal, as highlighted by the arrowheads. In inflammatory cuffs in the cerebellum of an EAE mouse, specific MMP activity occurs beyond the glia limitans (blue) at sites of inflammatory cell infiltration (red).

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Graphical representation of injection sites for EAE induction.** 1) Injection site for 30  $\mu$ L MOG-emulsion into the right flank, 2) injection site for 30  $\mu$ L MOG-emulsion into the left flank, 3) injection site for 20  $\mu$ L MOG-emulsion into the left tail root, 4) injection site for 30  $\mu$ L MOG-emulsion into the right tail root, 5) injection site for a little droplet of MOG-emulsion into the neck.

**Figure 2: Representative course of EAE.** (A) Disease symptoms of EAE in C57BL/6 mice over time. Mean +/- SEM is shown. (B) Change of body weight during EAE over time. Mean +/- SEM is shown.

**Figure 3: Representative picture of in vivo permeability.** (A) Left picture: green fluorescent 10

kDa dextran at the level of the choroid plexus. Middle picture: red fluorescent 3 kDa dextran at the level of the choroid plexus. Right picture: Merge of the left and middle pictures. Scale bar 50  $\mu$ m. (B) Left picture: green fluorescent 10 kDa dextran in the mouse cerebellum. Middle picture: red fluorescent 3 kDa dextran in the mouse cerebellum. Right picture: merge of the left and middle pictures. Dashed lines are indicative for blood vessel walls; arrow heads point to fluorescent tracers in the CNS parenchyma. Scale bar 100  $\mu$ m.

**Figure 4: Representative pictures of *in situ* zymography.** First column: green fluorescent channel (unquenched signal of dye-quenched (DQ) gelatin). Second column: blue fluorescent channel (laminin). Third column: red fluorescent channel (CD45). Fourth column: merge of fluorescent channels. Upper row: cold gelatin (non-fluorescent) negative control. Middle row: phenantroline (MMP-inhibitor) negative control. Lowest row: DQ gelatin. Scale bar 100  $\mu$ m.

**Table 1: Scoring criteria for the assessment of EAE disease severity.**

## DISCUSSION:

Here, we present a protocol to induce and monitor EAE in C57BL/6 mice. To assess the severity of EAE, we made use of a 3-point scoring sheet. EAE severity is generally scored with respect to the severity of motor dysfunctions. There are several scoring routines employed, reaching from 0-3 points to 0-6 points<sup>15-18</sup> or even more subpoints<sup>19</sup>. However, we have previously shown that supposedly refined EAE scoring does not lead to any improvement in determining potential statistically significant differences in disease scores between groups, when considering the overall disease severity<sup>14</sup>. Based on this observation we made use of the 3-point scale to assess disease progression in EAE in order to reduce mouse handling time and thus, distress for mice implementing the 3R rules. Another critical point using the EAE model to study differences between knockout and wild-type mice or differences between treatment groups is setting up a precise experimental planning. When wild-type mice are compared to knockout mice, it is essential to compare littermates originating from heterozygous matings to ensure identical genetic backgrounds of the wild-type and knockout mice compared in the EAE experiment. It is also important to keep the same conditions for all mice included in an experiment, e.g. cage changes, administration of wet food, and especially mouse housing (health state) conditions. In order to avoid cage-specific phenomena induced by the investigator, cross-immunization should be performed. More precisely, if more than one syringe is needed to immunize a certain number of mice, they should be randomly assigned to different syringes used. The latter likewise should be applied for the performance of treatment studies. Last but not least, the health status of mice housed in different animal facilities might impact on disease incidence and severity and thus more<sup>20</sup> or less *Mycobacterium tuberculosis*<sup>21</sup> might be required to induce a proper clinical disease. Furthermore, the site of injection may impact on disease development. In this protocol we propose to inject relatively small volumes into five different subcutaneous sites: 30  $\mu$ L into two flanks, 20  $\mu$ L into the left and right tail root, and a little droplet into the neck. In other protocols, 50  $\mu$ L depots are placed subcutaneously into the tail root only<sup>22</sup> or into the four flanks<sup>20</sup>. Injections of smaller depots, however, minimize the risk of causing skin irritation, which mice may try to scratch.

In vivo permeability might be performed to assess BBB leakage during EAE but also to assess whether the specific genetic deletion of a molecule or a drug treatment impacts on BBB integrity in vivo. This can be performed either by the detection of endogenous plasma tracers such as IgG deposits<sup>12</sup> or exogenous tracers that are applied into the circulation of an alive mouse<sup>23</sup>. In this protocol we made use of two different exogenous tracers (3 kDa and 10 kDa Dextran) that were simultaneously injected and allowed to circulate for a defined time of 15 min. Using two tracers with different sizes allows to determine the severity of the BBB dysfunction and may allow to define a cutoff for tracer sizes that diffuse or not across the BBB. Fluorescent dextrans are hydrophilic polysaccharides characterized by good water solubility, low toxicity, and relatively low immunogenicity. Furthermore, dextrans are composed of poly-( $\alpha$ -D-1,6 glucose), which are resistant to cleavage by endogenous cellular glycosidases. We here used dextran variants that have lysine residues incorporated into the dextran conjugate. Lysinated dextrans are aldehyde fixable, which allows to fix the tracer in the tissue. Besides fluorescently labeled dextrans, other chemicals can be used to assess in vivo permeability, e.g., Hoechst dye in combination with Evan's blue, which binds to a large part to serum albumin. Hoechst stains the BBB endothelial cell nuclei lining CNS microvessels, and Evan's blue can be detected in the perivascular space upon BBB dysfunction and diffuses further when the glia limitans is disturbed<sup>24, 25</sup>. In contrast, assessing in vivo permeability by immunofluorescent staining of endogenous markers provides insight into accumulation of, e.g., IgG molecules or fibrinogen, a glycoprotein that circulates in the blood, over time. In healthy conditions, the perivascular space is very narrow and the endothelial basement membrane and the glia limitans are closely connected and, thus, visualizing the two ECM barriers of the NVU requires laminin isoform-specific antibody staining<sup>26</sup>.

Endogenous IgG molecules are present during health and disease inside the blood vessels as well as in the tissue of circumventricular organs, where no BBB is established<sup>12</sup>. In gene modified mice with brain barrier defects, during drug treatment, or during neuroinflammation endogenous molecules that normally circulate in the blood might be deposited in the CNS parenchyma, which can be visualized by counterstaining with anti-laminin antibodies either recognizing all laminin isoforms (pan-laminin antibodies) or laminin isoform-specific antibodies allowing to differentiate laminins, laminin  $\alpha$ 4 and  $\alpha$ 5 from the endothelial basement membrane and laminin  $\alpha$ 1 and laminin  $\alpha$ 2 from the glia limitans. During neuroinflammatory conditions the perivascular space might be widened by infiltrating immune cells allowing to identify both, the endothelial basement membrane and the glia limitans using a pan-laminin antibody. Counterstaining of laminins present at the NVU allows to assess whether endogenous but also exogenous tracers are deposited in the CNS parenchyma and counterstaining of CD45 allows to assess whether focal leakage of the BBB is associated with immune cell infiltration.

Immune cell migration into the CNS parenchyma requires the sequential crossing of two distinct barriers within the NVU, the endothelial BBB, and subsequently the glia limitans<sup>27</sup>. Crossing the glia limitans and CNS entry of immune cells correlates with the onset of clinical EAE<sup>28</sup>, while CNS infiltrating cells trapped in leptomeningeal and perivascular spaces do not trigger clinical EAE. Immune cell trapping in leptomeningeal and perivascular spaces between basement membranes has been observed in several gene-modified mouse models e.g. L-selectin knockout mice<sup>29</sup>, TNF

knockout mice<sup>22</sup>, MMP2/MMP9 double knockout mice<sup>10</sup>, and JAM-B knockout mice<sup>12</sup>. These findings underscore that lack of these molecules does not affect immune cell migration across the BBB but rather across the glia limitans, underscoring that molecular mechanisms mediating immune cell migration across the BBB and the glia limitans are distinct. Brain *in situ* zymography is a methodology to investigate for focal gelatinase activity in a tissue section in precise spatial correlation to CNS pathology. Detection of *in vivo* BBB permeability together with performing *in situ* zymography to detect MMP2/MMP9 activity thus allows to delineate endothelial versus glia barrier disturbance in correlation to immune cell infiltration. Combined with pan-laminin and CD45 immunofluorescence staining on EAE brain sections, it allows both localization of gelatinase activity by unquenching DQ-gelatin and localization of infiltrating immune cells at the same time. Unquenching of fluorescein from DQ-gelatin results in a typical granular bright green fluorescence signal, while patchy dimly green fluorescent areas on tissue sections have to be considered as unspecific signal<sup>12</sup>. Thus, careful evaluation of tissue sections and proper controls are indispensable when performing *in situ* zymography. In this protocol, we performed *in situ* zymography in combination with CD45 and laminin staining. Primary antibodies against both CD45 and laminin were incubated as a mix at the same time. Similarly, secondary antibodies were incubated as a mix in a second staining step. Such a combination of antibodies needs testing to ensure that second stage antibodies are absorbed against other IgGs in order to avoid cross-reactivity.

Taken together, the protocols presented here provide tools to investigate in detail which element of the NVU is impaired in neuroinflammatory conditions of EAE. *In vivo* permeability assessment by exogenous tracers allows to identify current impairment of microvascular endothelial cell integrity or current impairment of the NVU. In addition, introduction of tracers with different sizes enables to estimate the severity of BBB impairment. Additional *in situ* zymography uncovers focal gelatinase activity in astrocytes and potentially inflammatory cells, which is needed to breach the glia limitans as a last barrier before getting access to the CNS parenchyma<sup>10</sup>.

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#### DISCLOSURES:

No conflicts of interest declared.

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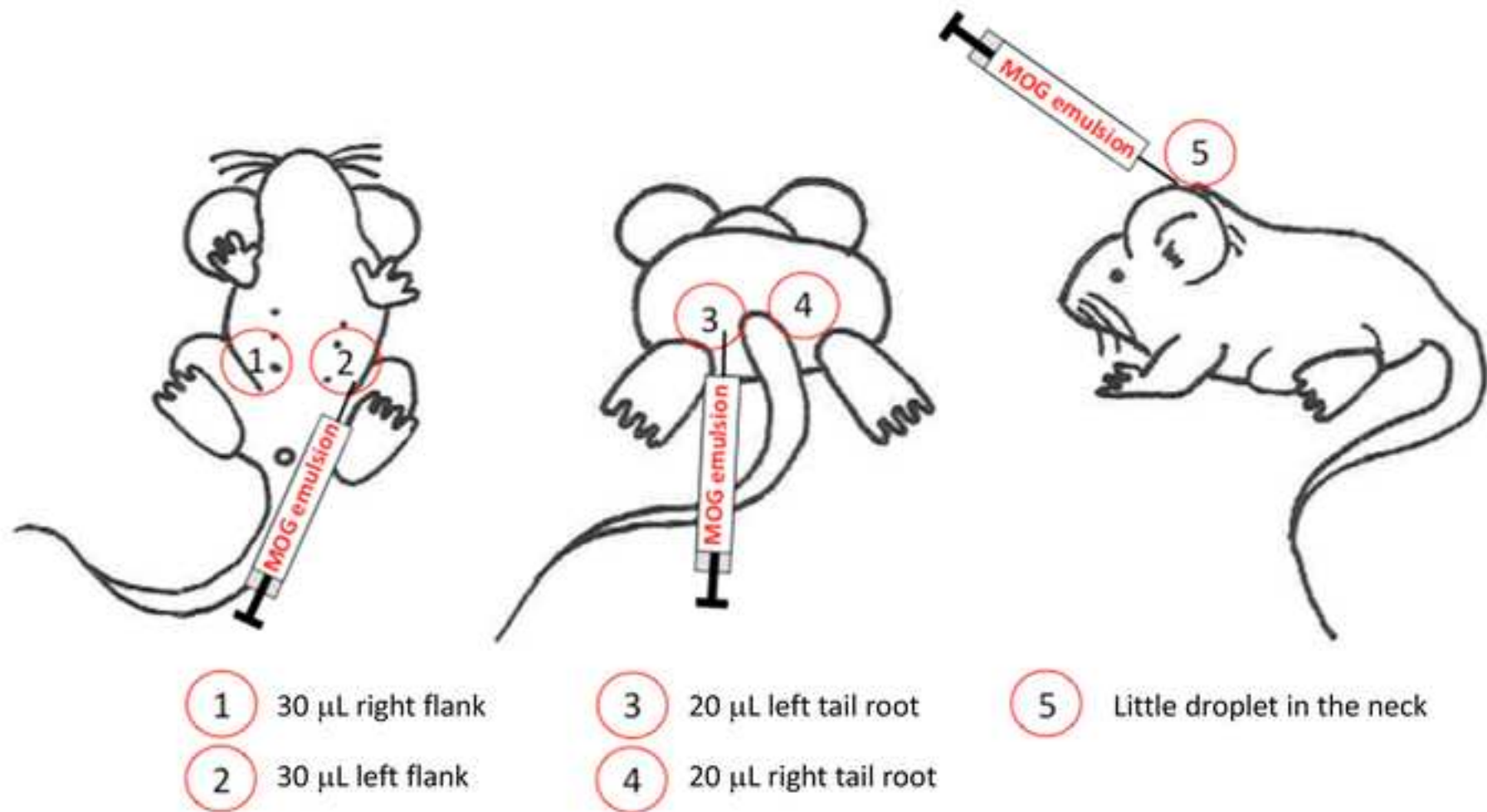
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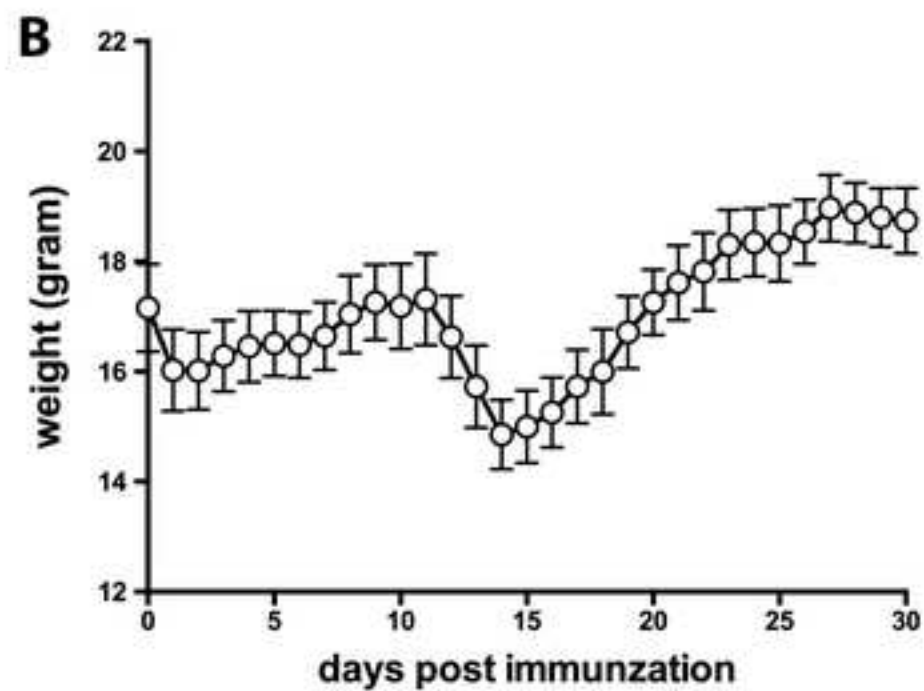
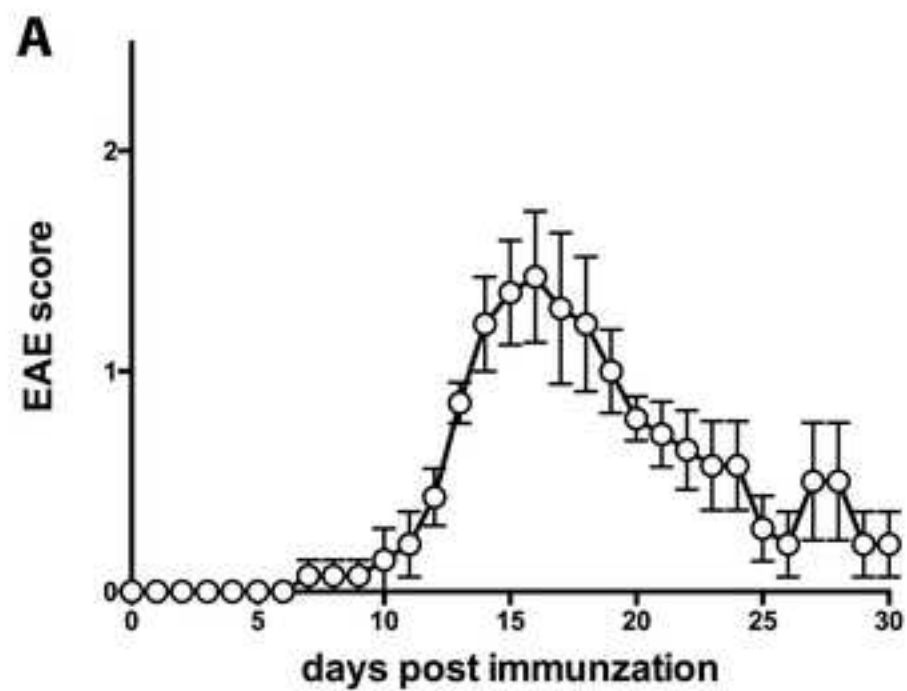
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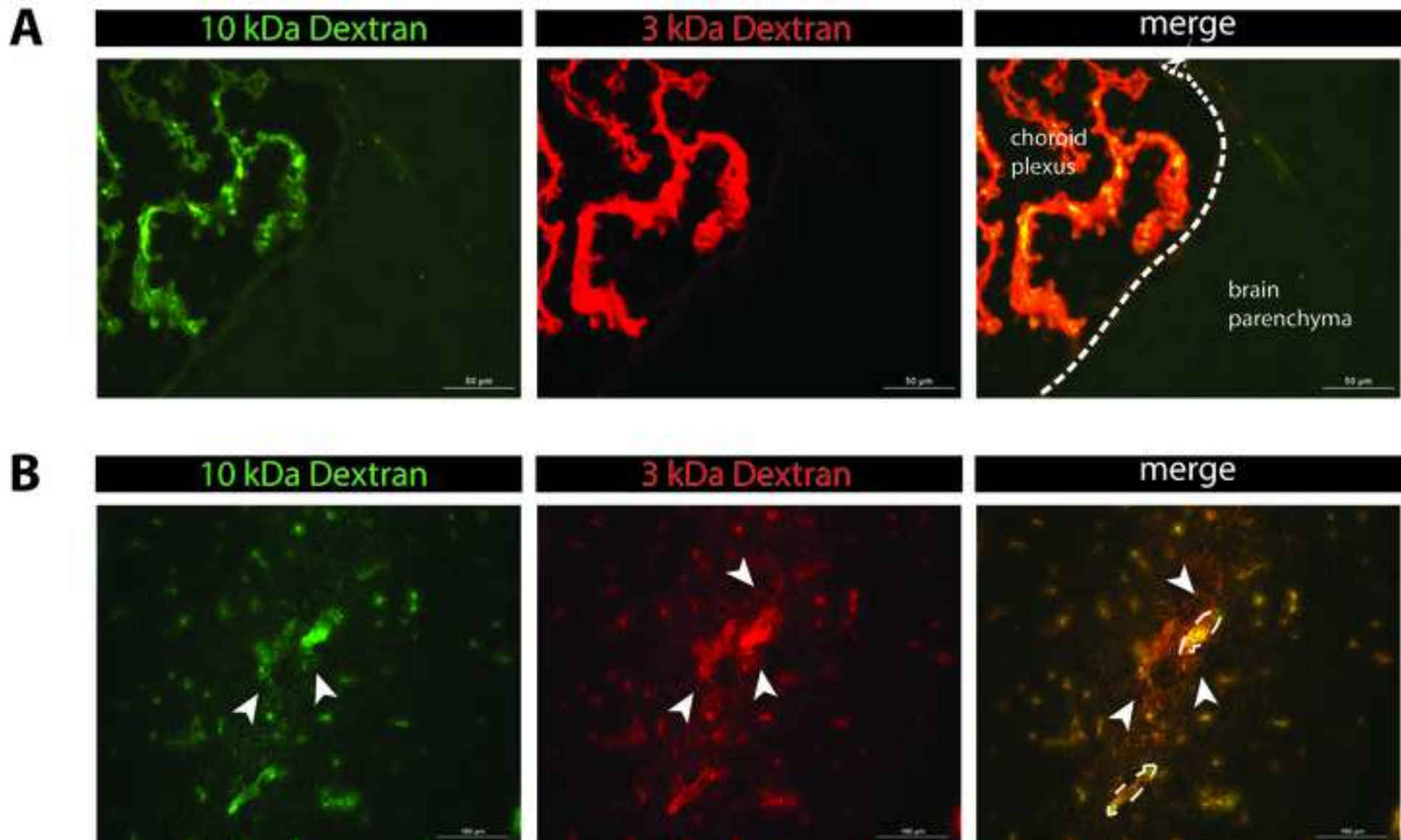
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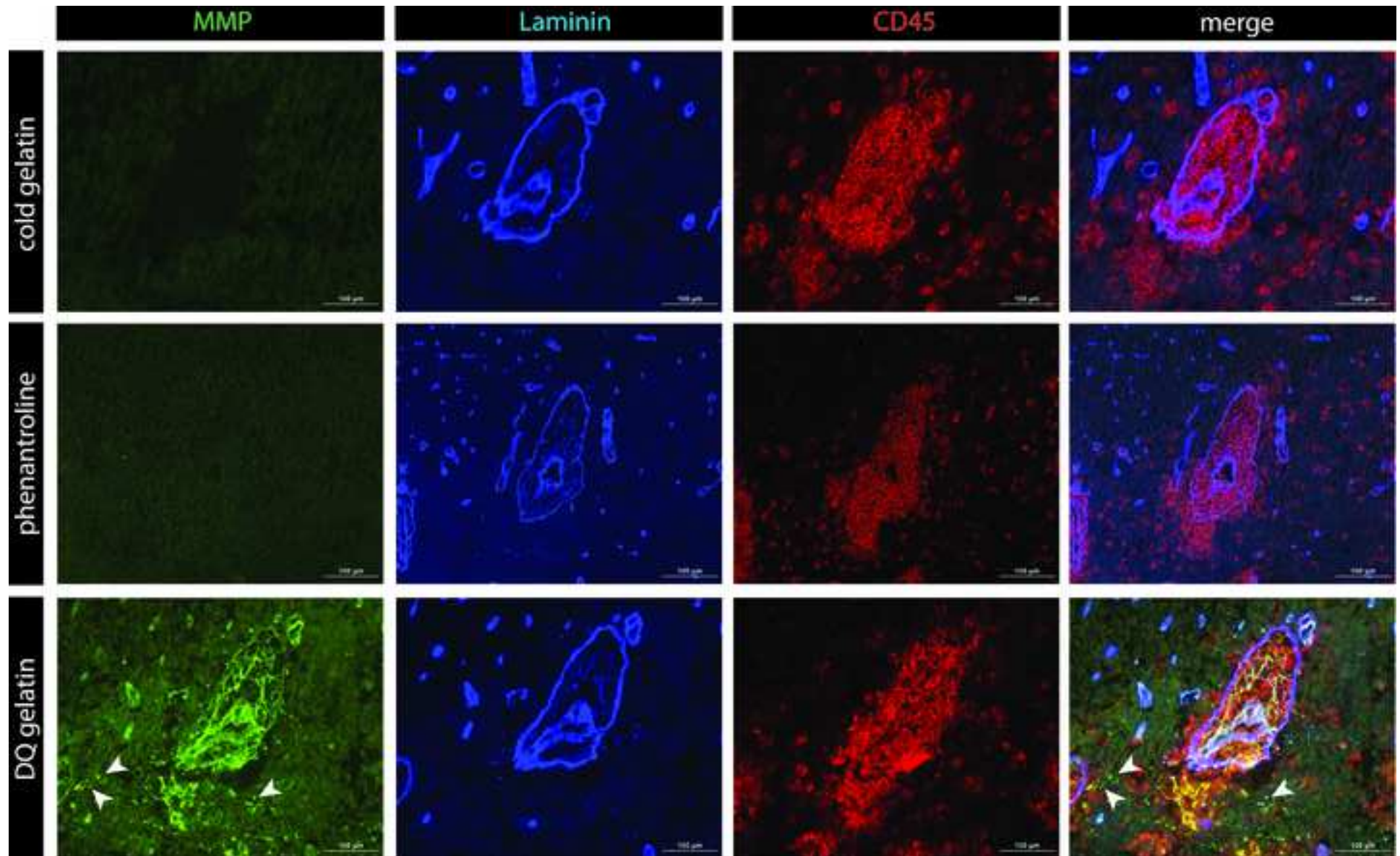
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**0 to 3 point score**  
**0**  
**~**  
**0.5**  
**1**  
**2**  
**3**

## **criteria**

**no signs**

weak tail (weak tonus) - inhouse criteria: NOT included in documented scoring

**limp tail** (tail drops)

**hindleg weakness, unsteady gait** (mouse walks on the bench like a duck)

**hindleg paraplegia** (mouse does not move its hindlimbs)

**hindleg paraplegia, incontinence loss of lower body control** (mouse falls to one side but can move in the c

### **weight change**

normal weight

weight loss (indicative for 2nd check)

weight loss

weight loss

severe weight loss (indicative for 2nd check)

very low body weight (indicative for 2nd check)



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
AMCA anti-rabbit antibody	Jackson ImmunoResearch	111-156-045	Store at 4 °C; protect from light
Anti-CD45 Antibody (30F11)	Pharmingen	07-1401	Store at 4 °C
Anti-Laminin Antibody	DAKO	Z0097	Store at 4 °C
Breeding food	e.g. PROVIMI KLIBA SA	3336	
BSA fraction V	Applichem	A1391	Store at 4 °C
Cold gelatine	Sigma-Aldrich	G 9391	
Coplin jar + rack	e.g. Carl Roth GmbH + Co. KG	H554.1; H552.1	
Cy3 anti-rat antibody	Jackson ImmunoResearch	111-156-144	Store at 4 °C; protect from light
Cover slips 24 x 40 mm # 1	e.g. Thermo Scientific	85-0186-00	
Dextran Alexa Fluor 488 (10,000 MW)	e.g. Molecular probes	D22910	Store at -20 °C; protect from light
Dextran Texas Red (3000 MW)	Invitrogen	D3328	Store at -20 °C; protect from light
EnzChek Gelatinase/Collagenase Assay Kit	Thermo Fisher Scientific; EnzCheck	E12055	Store at -20 °C; protect form light
Female C57BL/6J mice (8-12 weeks)	e.g. Janvier Labs		Females, 8-12 weeks
Freezing box for histology slides	e.g. Carl Roth GmbH + Co. KG	2285.1	
18G x 1½'' (1.2mm x 40mm) injection needle	e.g. BD, BD Microlance 3	304622	
27G x ¾'' – Nr. 20 (0.4mm x 19mm) injection needle	e.g. BD, BD Microlance 3	302200	
30G x ½'' (0.3 mm x 13 mm) injection needle	e.g. BD, BD Microlance 3	304000	
Incomplete Freund’s adjuvant (IFA)	e.g. Santa Cruz Biotechnology	sc-24648	Store at 4°C
Maintenance food	e.g. PROVIMI KLIBA SA	3436	
MOGaa <sub>35-55</sub> peptide	e.g. GenScript		Store at -80 °C

microscope slides (Superfrost Plus <i>Mycobacterium tuberculosis</i> H37RA NaCl 0.9 % O.C.T. compound (Tissue-Tek ) Omnican 50 30G x ½'' Paraformaldehyde Pertussis toxin	Thermo Scientific e.g. BD  B. Braun Sakura B. Braun Merck e.g. List biological laboratories, Inc.	J1800AMNZ 231141  3535789 4583 9151125S 30525-89-4 180	Store at 4 °C      Store at 4 °C
poly(vinyl alcohole) (Mowiol 4-88)	Sigma-Aldrich	81381	
Protease Inhibitor EDTA free repelling pen e.g. DAKO Pen sealing film e.g. Parafilm M Silica gel Stitch scissor syringe 1 ml syringe 10 ml vaporizer system Univentor 400	Sigma-Aldrich e.g. DAKO e.g Sigma-Aldrich e.g. Carl Roth GmbH + Co. KG F.S.T e.g. PRIMO e.g. CODAN Medical ApS UNO.BV	4693132001 S2002 P7793 9351.1 15012-12 62.1002 2022-05	Store at 4 °C

Tissue-Tek Cryomold 25 x 20 x 5  
vaporizer system (Univentor 400)

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### CORRESPONDING AUTHOR:

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Signature:		Date:	2018/15/10

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## Point-by-point reply

### **Manuscript: 59249\_RO\_101618 entitled "Visualizing *in vivo* blood-brain barrier permeability and gelatinase activity in brain sections of mice with experimental autoimmune encephalomyelitis"**

Silvia M Tietz<sup>1</sup>, Britta Engelhardt<sup>1</sup>

<sup>1</sup>Theodor Kocher Institute, University of Bern, Bern, Switzerland

Dear Editor and dear Reviewers

Please find our point-by-point answers to your queries below:

#### **Editorial comments:**

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*Answer: this has been done*

2. Figure 3: Please replace commercial language "DQ" with a generic term.

*Answer: We have used now the generic term "dye-quenched gelatine".*

3. Please shorten the title if possible.

*Answer: this has been done*

4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

*Answer: This has been changed as requested.*

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Eppendorf, cOmpete™, DQ™, Tissue-Tek, Superfrost®, Mowiol, EnzChek™, etc.

*Answer: This has been addressed accordingly.*

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

7. 2.1.3: Please split into two steps.

*Answer: this has been done.*

8. 2.2.1: Please specify the concentration of isofluorane and mention how proper anesthetization is confirmed.

*Answer: This information has been added.*

9. 2.2.5: Please describe the composition of maintenance diet and breeding diet.

*Answer: this information has been included*

10. 4.6: What volume of PFA is used?

*Answer: Has been added.*

11. 4.7: Please specify the surgical instruments used.

*Answer: We have rephrased this paragraph.*

12. 6.6: What is used to cut tissue sections?

*Answer: This information has been added.*

13. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

*Answer: This has been addressed.*

14. Please include single-line spaces between all paragraphs, headings, steps, etc.

*Answer: this has been done.*

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

*Answer: this has been done*

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

*Answer: this has been respected.*

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

*Answer: this has been done*

18. Discussion: Please discuss any limitations of the technique.

*Answer: this has been done.*

19. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

*Answer: this has been done*

20. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below: Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).



*Answer: addressed*

21. References: Please do not abbreviate journal titles. If there are six or more authors, list the first author and then "et al."

*Answer: addressed*

22. Table of Materials: Please sort the items in alphabetical order according to the Name of Material/Equipment.

*Answer: This has been addressed*

## **Reviewers' comments:**

### **Reviewer #1:**

Manuscript Summary:

Engelhardt and Tietz present valuable methodologies for neuroimmunological research. The way of presentation is clear and contains sufficient details. I am convinced that video that is going to be produced will complement the manuscript perfectly.

Major Concerns:

none

Minor Concerns:

There are some parts of the text that are highlighted. I am not aware of the purpose of the highlighting, but these are essential parts of the protocols, and they should stay in the final version of the manuscript.

*Answer: This is the part of the manuscript highlighted on request of the journal to be considered for making the movie.*

"end-feed" should be replaced with "end-feet" throughout the manuscript.

*Answer: has been corrected*

There are other typos and grammar errors in the manuscript, please check carefully.

*Answer: has been corrected*

Please, explain "breeding diet" and its purpose regarding EAE induction.

*Answer: This information has been included*

### **Reviewer #2:**

Manuscript summary:

In this manuscript Tietz and Engelhardt describe detailed protocols to investigate the impairment of the neurovascular unit in an animal model of neuroinflammation. They describe:

1. how to induce EAE in C57BL/6 mice by active immunization,
2. how to analyze blood brain barrier permeability by using a combination of exogenous fluorescent tracers of two different molecular weights.
3. A protocol for combining in situ zymography and immunofluorescence for simultaneous detection of gelatinase activity and immune cell infiltration across the basement membranes.

The manuscript is well-written, well-organized and for the most part clearly presented. In particular, the authors should be commended for explaining their rationale for using these assays in the particular way and combination presented in this manuscript, specific reagents for their chemical, non-immunogenic, or other properties, and the importance of the accomplished scientific observations for deciphering underlying biological events.

However, a few issues still need to be addressed that should substantially improve the impact of this

publication:

Major issues:

1. Representative results need to be provided for the BBB permeability assay comparing uninjured with EAE conditions, and clearly demonstrating how this protocol detects BBB disruption and why the combination of these two molecular weight tracers is advantageous. Fig.2 currently does not demonstrate leakage in the CNS and does not clearly explain what the advantage of using these two tracers in combination is.

*Answer: A revised figure has been included to address this request accordingly.*

2. In addition to the instructions provided on how to most efficiently make essential solutions, the concentrations of final and intermediate solutions should also be provided for all protocols.

*Answer: This information has been included.*

3. 2.2 EAE induction: point 2.2.3: The authors should detail the different injection spots with the exact volumes used here and not in the discussion. A schematic would also help if the authors would not mind preparing one.

*Answer: A schematic figure has been included*

4. The EAE scoring scale should be better presented and explained so an inexperienced experimenter can learn what signs to look for. The weight correlation is rather confusing too. What is the meaning of "indicative of second check"? It currently appears on two pages with missing text. Details regarding when they start to weigh and score would also help.

*Answer: This has been explained in more detail in the revised manuscript. Please note that each explanation in the text will be correlated to a video showing exactly what to look for.*

5. 4-In vivo permeability assay: point 4.6: The protocol needs to be more clear regarding fixation, post-fixation and cryopreservation of the tissue (with sucrose?)

*Answer: this has now been addressed in paragraph 5. Please note that in situ zymography is not performed on the same tissue sections analyzed for BBB permeability*

6. 7-In situ zymography: point 7.9: If the sections are already fixed with PFA, do they need to be fixed again with methanol? This seems to be in contrast to what the authors describe in their recently published JAMB in EAE study.

*Answer: Please our explanation to point 5.*

7. Is a permeabilization reagent used during the washes or the incubation with the primary or secondary antibodies?

*Answer: No permeabilization reagent is used during the washes or the incubation with primary or secondary antibodies.*

Minor issues:

1. The language needs some professional editorial work to improve readability and clarity, and correct some typos and grammatical errors

*Answer: this has been addressed.*

2. The numbering of steps needs to be checked for consistency

*Answer: this has been addressed.*

3. Abbreviations should always follow the terms they are meant to abbreviate (ex: ECM)

*Answer: this has been addressed.*

4. 2.1.3: Clarify the language: adjust the emulsion volume?

*Answer: done.*

5. Explain the rationale behind the diet change in EAE mice

*Answer: this information has been included.*

6. 4.6 the sentence is confusing. Clarify the order of steps and how the two syringes are used and what for (not for opening the atrium presumably)

*Answer: this has been addressed.*

7. 5.7 place brain slices with the front facing down should be rephrased to the anterior side of each brain pieces you cut in a previous step

*Answer: This has been changed.*

8. 6-Preparation of frozen tissue sections: point 6.1: describe for how long should pre-warming the frozen tissue in the cryostat last

*Answer: this has been included.*

9. 6.4 clarify the parenthesis

*Answer: The explanation has been added to now paragraph 7.2.*

10. Some additional references for original findings discussed throughout the manuscript and particularly in the discussion could be included for proper scientific etiquette

*Answer: We have added additional references to address original findings on investigating BBB integrity in vivo using FITC-albumin (KRUEGER 2015) and Evans Blue (GRAESSER 2002).*