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TITLE:

Quantitative Measurement of Intrathecally Synthesized Proteins in Mice

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KEYWORDS:

intrathecal synthesis, cerebrospinal fluid, blood-brain barrier, albumin quotients, protein index, protein transudate

SUMMARY:

Elevated spinal fluid protein levels can either be the result of diffusion of plasma protein across an altered blood-brain barrier or intrathecal synthesis. An optimized testing protocol is presented in this article that helps to discriminate both cases and provides quantitative measurements of intrathecally synthesized proteins.

ABSTRACT:

Cerebrospinal fluid (CSF), a fluid found in the brain and the spinal cord, is of great importance to both basic and clinical science. The analysis of the CSF protein composition delivers crucial information in basic neuroscience research as well as neurological diseases. One caveat is that proteins measured in CSF may derive from both intrathecal synthesis and transudation from serum, and protein analysis of CSF can only determine the sum of these two components. To discriminate between protein transudation from the blood and intrathecally produced proteins in animal models as well as in humans, CSF protein profiling measurements using conventional protein analysis tools must include the calculation of the albumin CSF/serum quotient ($Q_{albumin}$), a marker of the integrity of the blood-brain interface (BBI), and the protein index ($Q_{protein}/Q_{albumin}$), an estimate of intrathecal protein synthesis. This protocol illustrates the entire procedure, from CSF and blood collection to quotients and indices calculations, for the quantitative measurement of intrathecal protein synthesis and BBI impairment in mouse models

of neurological disorders.

INTRODUCTION:

Cerebrospinal fluid (CSF), a clear and colorless liquid surrounding the brain and the spinal cord, holds great clinical and basic scientific importance. The CSF preserves the electrolytic environment of the central nervous system (CNS), balances the systemic acid-base status, supplies nutrients to neuronal and glial cells, functions as a lymphatic system for the CNS, and transports hormones, neurotransmitters, cytokines and other neuropeptides throughout the CNS[1]. Thus, as the CSF composition reflects the activity of the CNS, this fluid offers a valuable, though indirect, access to characterize the physiological and pathological state of the CNS.

CSF has been used to diagnose conditions that affect the CNS for over a hundred years, and for most of this time, it was primarily studied by clinicians as a diagnostic tool. However, in recent years neurobiologists have recognized the potential of CSF for studying the pathophysiology of the CNS. In particular, several high-throughput protein analysis tools have been introduced in the neuroscience realm allowing a detailed study of the protein composition of the CSF, with the expectation that this analysis may help provide insight into the dynamic changes occurring within the CNS.

Technological developments in multiplex immunoassay techniques such as Luminex and Simoa technologies[2,3], provide researchers today with the ability to detect hundreds of proteins at very low concentrations. Moreover, these same technologies allow the use of small sample volumes, thereby promoting studies in small animals, including mice, in which limited sample volumes of CSF has precluded detailed characterizations of the fluid until recently.

Nevertheless, one caveat is that proteins measured in CSF may derive from intrathecal synthesis and/or transudation from serum due to a damaged blood-brain interface (BBI). Unfortunately, protein analysis of CSF alone can only determine the sum of these two components. To discriminate between transudate and intrathecally produced proteins, CSF protein measurements using any available protein analysis tool must be adjusted for individual variability in serum concentrations as well as barrier integrity. However, although this adjustment is commonly used in clinical practice, e.g., the CSF IgG index, which has high sensitivity for detecting intrathecal IgG synthesis[4-6], to date very few research studies have corrected CSF protein concentrations for serum concentration and barrier integrity[7,8].

Currently, the Reibergram approach is the best way to determine the barrier function and intrathecal synthesis of proteins. It is a graphical evaluation in CSF/serum quotient diagrams which analyzes, in an integrated way, both the barrier (dys)function and intrathecal protein synthesis, referring to an exclusively blood-derived protein[9,10]. The highly abundant protein albumin is usually chosen as reference protein because it is produced only in the liver and because its size, approximately 70 kDa, is intermediate between small and large proteins[11]. The analysis diagram was first defined by Reiber and Felgenhauer in 1987 for the major classes of immunoglobulins (Igs)[11], being empirically based on the results obtained from the analysis of thousands of human samples[9]. The approach was subsequently confirmed by the application of

the two Fick's laws of diffusion in the theory of molecular diffusion/flow rate[12]. Such a theory demonstrates the diffusion of a protein through the barrier has a hyperbolic distribution and can quantitatively explain the dynamics of proteins in the CNS[9,13]. Overall, the advantage of using the Reibergram for demonstrating intrathecal protein synthesis is that it concurrently identifies the protein fraction that enters the CSF from serum as well as the amount of protein found in the CSF because of local production.

The present article and the related protocol describe the entire procedure, from CSF and blood collection to the final calculations correcting CSF protein levels, for the quantitative measurement of intrathecal protein synthesis in mouse models of neurological disorders. This procedure provides a baseline against which to assess (1) the pathophysiological origin of any CSF protein and (2) the stability and functional significance of the barrier integrity. This procedure and protocol are not only useful for assessing mouse CSF samples but are also useful in analyzing CSF in a multitude of animal models of neurological diseases and human patients.

PROTOCOL:

All animal work utilizes protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Geisel School of Medicine at Dartmouth.

1. Collection of fluids

NOTE: Both serum and CSF are required. Two protocols for each fluid collection are needed for survival and necropsy.

1.1. Serum and CSF collection using survival procedures

NOTE: For survival fluid collection, serum collection should precede CSF collection as it is a less invasive procedure. CSF must be obtained within one week of serum draw.

1.1.1. Retro-orbital bleeding procedure for serum collection.

NOTE: This procedure is for survival bleeding of mice[14]. The procedure described applies to any age, gender, and strain of mice. Since IACUC rules dictate that a maximum blood volume of 1% of body weight can be removed as a single blood draw, it is recommended the procedure is performed only on mice weighing more than 15 g.

1.1.1.1. Move the cages containing mice from the rack to an appropriate working area. Prepare the anesthesia gas machine by turning on the oxygen flow meter to 1 L/min.

1.1.1.2. Place the animal into the induction chamber and close the lid tightly. Turn on the isoflurane vaporizer to 3.5% and monitor the animal until recumbent.

1.1.1.3. Remove the animal from the chamber and assess the level of anesthesia by pedal reflex,

i.e., firm footpad pinch. Ensure adequate depth of anesthesia before performing the procedure: lack of response to a firm pinch indicates adequate anesthesia.

1.1.1.4. Restrain the anesthetized mouse by grasping the loose skin behind the ears with the thumb and index finger of the non-dominant hand. Bulge the eyes by using the index finger to draw back the skin above the eye and the thumb to draw back the skin below the eyes.

1.1.1.5. Place the tip of a Pasteur pipette into the eye socket underneath the eyeball (**Figure 1**, left panel), directing the tip at approximately 45° toward the middle of the eye socket (**Figure 1**, right panel). Rotate the pipette between fingers during the forward passage. Apply gentle pressure and then release until blood is entering the pipette.

NOTE: Maximum amount of blood that may be withdrawn at one time from this location is about 1% of body weight, e.g., 0.2 mL from a 20 g mouse.

1.1.1.6. Gently remove the capillary to prevent injury to the eye and place the collected blood in a 1.5 mL centrifuge tube. Close the eyelid and apply mild pressure with gauze to prevent further bleeding. Once fully alert and mobile (usually 3−5 min), return the mouse to its holding cage.

1.1.1.7. Allow blood to clot for 30−60 min at room temperature (RT), then centrifuge blood for 10 min at 2,000 x *g* in a 4 °C refrigerated centrifuge. Using a clean pipette technique, collect serum into a new, labeled 0.5 mL vial. Immediately freeze vial of serum at -80 °C.

1.1.2. CSF collection with survival procedure

NOTE: This procedure is for survival surgery, and it is based on the protocol published by Liu and Duff in 2008[15]. The mice are anesthetized by a Ketamine (20 mg/mL), xylazine (0.5 mg/mL), and acepromazine (0.5 mg/mL) cocktail administered intraperitoneally.

1.1.2.1. Move the cages containing mice from the rack to a designated surgery working area. Prepare surgery space in a sterile environment. Ensure that all instruments and materials used are sterilized before surgery.

1.1.2.2. Weigh the mouse and calculate the anesthesia volume needed (0.1 mL of anesthesia cocktail for a 20 g mouse). Inject anesthesia intraperitoneally[16]. After a few minutes, test the mouse by pinching the footpad to ensure adequate anesthesia. If more anesthetic is required, further inject 0.01−0.03 mL of the anesthetic cocktail.

1.1.2.3. Use either scissors or a shaver to shave a small area of the head, on the caudal end, medial on the skull, to expose large enough working area for CSF collection. Position the mouse in the prone position on the stereotaxic instrument, and steady the head by using ear bars (**Figure 2A**).

NOTE: The mouse is laid down so that the head forms a nearly 135° angle with the body (**Figure**

2A).

1.1.2.4. Swab the surgical site with 30% chlorhexidine diacetate. Using surgical scissors, make a sagittal incision of the skin inferior to the occiput to expose muscles overlying the cisterna magna.

1.1.2.5. By blunt dissection with forceps, separate the subcutaneous tissue and muscles to expose the cisterna magna (**Figure 2B**). Use microretractors to hold the muscles apart (**Figure 2B**) and expose the dura mater meningeal layer over the cisterna magna.

1.1.2.6. Gently wash with sterile phosphate-buffered saline (PBS) to remove any possible blood contamination. Blot dry the dura mater with a sterile cotton swab and gently puncture the membrane covering the cisterna magna with a 30 G needle. Quickly and gently insert a small glass capillary tube to collect CSF (**Figure 2C**).

NOTE: Intracranial pressure allows CSF to flow spontaneously into the capillary (**Figure 2C**). Depending on the age and size of the mouse, approximately 5−12 µL of CSF is obtained from each mouse.

1.1.2.7. Carefully remove the capillary tube from the membrane. Connect the tube to a 3 mL syringe through a polyethylene tubing (**Table of Materials**) and inject the collected CSF into a labeled 0.5 mL tube (**Figure 2D**). Keep vials in ice.

1.1.2.8. Close incision by using polydioxanone suture (PDS) with disposable needle and using buried sutures[17]. Clean off the area of any dried blood or tissue.

1.1.2.9. Inject mice, subcutaneously or intraperitoneally[16], with 0.05−0.1 mg/kg of buprenorphine hydrochloride as analgesic treatment. Also, inject subcutaneously 1 mL of sterile saline to prevent dehydration.

1.1.2.10. Place the mouse back in a clean and warm cage for recovery. Once the mouse is mobile and able to reach food and water, place the cage back on the rack.

1.1.2.11. Centrifuge CSF for 10 min at 1,000 x *g* in a 4 °C refrigerated centrifuge. Check the degree of blood contamination by visual inspection for identification of xanthochromia and presence of a red pellet in the bottom of the tube. Discard blood-contaminated samples.

NOTE: The formula utilized for the correction of CSF protein amounts in blood-contaminated specimens is based on equation parameters that include protein content in CSF and serum, hematocrit (HCT), and red blood cells (RBC) count in CSF and blood[18]. However, such a correction strategy cannot be easily applied to mouse CSF specimens due to the small volume, therefore limiting the correction strategy to a visual inspection.

1.1.2.12. Using a clean pipette technique, collect CSF into a new 0.2 mL tube, leaving behind the pellet with cells. Dilute CSF 1:3 with PBS to reduce volume loss due to aerosol. Immediately freeze

the vial of CSF at -80 °C.

1.2. Serum and CSF collection using non-survival procedures

NOTE: For non-survival fluid collection, CSF collection precedes serum collection as the mouse needs to have a pulse.

1.2.1. CSF collection at necropsy

NOTE: This procedure is for non-survival surgery, and approximately 10−20 µL of CSF is obtained from each mouse.

1.2.1.1. Move the cages containing mice from the rack to a comfortable working space. Follow steps 1.1.2.2−1.1.2.7 and 1.1.2.11−1.1.2.12 for CSF collection. Proceed to section 1.2.2 for serum collection.

1.2.2. Blood collection via intracardiac puncture (open approach)

NOTE: Blood volumes expected is approximately 3% of body weight, e.g., 0.6 mL from a 20 g mouse.

1.2.2.1. Following CSF collection ensure the mouse is still sufficiently anesthetized by pinching the footpad. If any reaction is observed, administer a second dose of anesthetic. If no reaction is observed, proceed.

1.2.2.2. Place the animal on the back and swab skin on the abdomen with 70% alcohol. With surgical scissors, open the thoracic cavity and expose the heart. Insert a 25 G needle (attached to a 3 mL syringe) into the left ventricle and gently apply negative pressure on the syringe plunger. Withdraw needle after blood has been collected.

1.2.2.3. Perform a secondary method of euthanasia such as decapitation or cervical dislocation to ensure that the animal is deceased.

1.2.2.4. Push the plunger of the syringe down and inject the collected blood into a 1.5 mL vial. Allow blood to clot for 30 min at RT and then centrifuge it for 10 min at 2,000 x *g* in a 4 °C refrigerated centrifuge.

1.2.2.5. Using clean pipette technique, collect serum into a new, labeled 0.5 mL vial. Immediately freeze vial of serum at a -80 °C freezer.

2. Protein analysis

2.1. Use a preferred method, e.g., Luminex technology, for quantifying target protein(s) and albumin in matched serum and CSF specimens.

NOTE: Here, an example is given with Luminex magnetic technology, but virtually any technique that measures protein amounts, including enzyme-linked immunosorbent assays (ELISAs), can be applied to the current protocol. Ideally, CSF and serum samples are run for both albumin and target proteins on the same platform. Assay conditions must be optimized for crucial steps in the protocol such as antigen-bead coupling concentration, serum and CSF sample dilutions, best-fit standard curves for each analyte, and buffer composition to reduce non-specific reactivity. If a commercial kit is used for protein(s) measurement, e.g., the immunoglobulin isotyping kit (**Table of Materials**) used to obtain data presented in **Figure 3**, manufacturers' instructions have to be followed.

2.1.1. Upon thawing and prior to analysis, centrifuge CSF and serum samples (2,000 x *g* for 10 min) and use the supernatant to prevent clogging of the filter plates and/or probe. Follow the assay procedure provided with the kit for appropriate sample dilutions. Otherwise, determine the appropriate dilution for each analyte and fluid. Dilute samples in PBS accordingly.

NOTE: If there are no specific guidance or instructions, dilutions for each analyte and fluid have to be established before the study test, by determining the appropriate dilution ranges necessary to obtain concentration estimates that fall within the most reliable range of a standard curve. Knowing the characteristics of the biological sample to be analyzed, e.g., physiological and pathological concentrations in the fluid, allows trying different dilutions with samples of low, medium, and high analyte content. If the expected range of concentrations in the samples is known a priori, the dilutions can be selected after calculating how many times the sample has to be diluted in order to be within the chosen standard curve range.

CAUTION: By calculating the dilution factors, remember that CSF has already been diluted 1:3.

2.1.2. Prepare a standard curve for each protein of interest, e.g., albumin and IgG as used to generate data in **Figure 3**, by serial diluting reference standard proteins. During the preparation of standard curves, thoroughly mix each higher concentration before making the next dilution.

NOTE: Regardless of the chosen method of quantification, it is essential to include a standard curve each time the assay is performed to estimate protein(s) concentration in samples. The best choice for a reference standard is a purified, known concentration of the protein of interest. Deciding on the specific dilutions, as well as the number of data points and replicates used to define the standard curve, depends upon the degree of non-linearity in the standard curve.

2.1.3. Select the appropriate antibody-coupled magnetic bead sets (**Table of Materials**). For individual vials of beads, sonicate each vial for 30 s and vortex for 1 min. Prepare a "working beads mixture" by diluting the bead stocks to a final concentration of 50 beads of each set/µL in assay/wash buffer (PBS, 1% bovine serum albumin [BSA]). Add 50 µL of the mixed beads to each well in a flat-bottom 96-well plate (**Table of Materials**).

CAUTION: The fluorescent beads are light-sensitive. Therefore, they should be protected from

prolonged exposure to light throughout the procedure.

2.1.4. Diagram the placement of backgrounds, standards, and samples on a well map worksheet.

2.1.5. Add 50 µL of assay/wash buffer to each background well, and 50 µL of each standard to the wells for the standard curve. Load 50 µL of each diluted sample into the appropriate wells last. Wrap the plate with foil and incubate with agitation (~800 rpm) on a plate shaker for 30 min at RT.

2.1.6. Place the plate on a handheld magnet (**Table of Materials**) and rest the plate on the magnet for ~60 s to allow complete setting of magnetic beads. Remove well contents by gently decanting the plate and tap plate on absorbent pads to remove residual liquid.

2.1.7. Wash the plate by removing it from the magnet, by adding 200 µL of assay/wash buffer, by shaking for ~30 s, and finally by reattaching it to the magnet. Repeat washing 3x.

2.1.8. Dilute the biotinylated detection antibody, i.e., biotin-labeled antibody raised against the protein host species, to 4 µg/mL in assay/wash buffer. Add 50 µL of the diluted detection antibody to each well. Cover the plate and incubate for 30 min at RT on the plate shaker at ~800 rpm. Place the plate on the magnet and repeat steps 2.1.6 and 2.1.7.

2.1.9. Dilute phycoerythrin (PE)-conjugated streptavidin (SAPE) to 4 µg/mL in assay/wash buffer. Add 50 µL of diluted SAPE to each well. Cover the plate and incubate for 30 min at RT on the plate shaker at ~800 rpm. Place the plate on the magnet and repeat steps 2.1.6 and 2.1.7.

2.1.10. Remove the plate from the magnet and resuspend the beads in 100 µL of assay/wash buffer. Read wells with a dual laser flow-based detection instrument which allows for the detection of the magnitude of PE fluorescence intensity (FI).

NOTE: The signal, e.g., FI, generated is proportional to the amount of target antigen attached to the surface of the beads.

2.1.11. Export raw data and create standard curves by graphing detection signal FI versus standard protein concentrations. Use the standard curve(s) to calculate the concentration of the analyte(s) in the samples.

NOTE: Albumin is preferentially expressed in g/dL, while proteins of interest are preferentially expressed in mg/dL.

3. Intrathecal index calculations

3.1. Organize protein concentration values into a spreadsheet and analyze the results by applying the following formulas.

3.2. Calculate $Q_{albumin}$:

$$Q_{albumin} = \left(\frac{CSF_{albumin}}{Serum_{albumin}}\right)$$

where $CSF_{albumin}$ and $Serum_{albumin}$ are concentrations of albumin in matched serum and CSF specimens, respectively.

3.3. Calculate $Q_{protein}$:

$$Q_{protein} = \left(\frac{CSF_{protein}}{Serum_{protein}}\right)$$

where $CSF_{protein}$ and $Serum_{protein}$ are concentrations of target protein(s) in matched serum and CSF specimens, respectively.

3.4. Calculate the protein index:

$$\text{Protein index} = \frac{Q_{protein}}{Q_{albumin}}$$

REPRESENTATIVE RESULTS:

This representative experiment aimed to compare the intrathecal synthesis of IgG in two clinically relevant rodent models of multiple sclerosis (MS): the $PLP_{139-151}$-induced relapsing experimental autoimmune encephalomyelitis (R-EAE) and the chronic progressive, Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD). R-EAE is a useful model for understanding relapsing-remitting MS, whereas the TMEV-IDD model features chronic progressive MS[19].

For the present study, a quantitative analysis of the intrathecal IgG synthesis in R-EAE (n = 12) and TMEV-IDD (n = 28) has been performed. Both groups were analyzed at the peak of their disease. An additional group of 10 mice was sham-treated and served as age-matched control groups (cR-EAE n = 4, cTMEV-IDD sham n = 6).

A magnetic bead-based approach with the commercially available kit (**Table of Materials**) was used to measure total IgG in matched serum and CSF specimens. The total IgG value was derived from the sum of the subclass IgG1, IgG2a, IgG2b, and IgG3 values. Albumin was measured with a commercial mouse albumin ELISA kit (**Table of Materials**) because a Luminex assay for albumin was not available at that time. All measurements were performed carefully following the manufacturers' instructions. Albumin quotient ($Q_{albumin}$) and IgG index ($Q_{IgG}/Q_{albumin}$) were then used to differentiate blood- versus CNS-derived IgG in the CSF.

As shown in **Figure 3A**, actual levels of total IgG are significantly increased in the CSF of both rodent models of MS when compared to the corresponding age-matched sham controls ($p \leq 0.026$). However, R-EAE mice show significantly enhanced $Q_{albumin}$ values ($p \leq 0.019$), indicating increased permeability of the barrier in these mice (**Figure 3B**). Conversely, no differences in $Q_{albumin}$ exist between TMEV-IDD and sham mice ($p = 0.49$), thus corroborating our previous

finding of an intact barrier in TMEV-IDD mice[7,8]. To further discriminate between transudate and intrathecally produced IgG in R-EAE and TMEV-IDD, the IgG Index was measured, showing significantly higher values in TMEV-IDD mice ($p \leq 0.0006$), and therefore intrathecal IgG production in this model (**Figure 3C**).

An intact barrier in TMEV-IDD mice along with a high IgG index suggests that in this model, antibody is produced within the CNS. Conversely, in R-EAE, a significant barrier breakdown and a low IgG index provide evidence that the CSF IgG is mostly produced by peripheral rather than intrathecal B cells, also suggesting that in this acute model of MS, CSF IgG is mostly derived from serum.

FIGURE LEGENDS:

Figure 1: Retro-orbital bleeding of mice. Left: Correct placement of the needle relative to the retro-orbital sinus, the eyeball and the back of the orbit. Right: Pipette location begins in the medial canthus of the eye and glides to the dorsal aspect of orbit. The capillary is inserted at an angle of 45°.

Figure 2: CSF collection in mice. (**A**) The ear bars support the head of the mouse, and the mouse is laid down so that the head forms a 135° angle with the body. The arrow points to the exposed cisterna magna. (**B**) By blunt dissection with forceps, the muscles are separated to expose the cisterna magna (pointed by the arrow). Microretractors are used to hold the muscles apart. (**C**) A small glass capillary tube is used to collect CSF from the cisterna magna. CSF flows spontaneously into the capillary, due to the intracranial pressure. The arrow points to the collected CSF in the capillary. (**D**) The CSF is transferred into a 0.5 mL tube through a modified 3 mL syringe.

Figure 3: Blood-brain barrier function and intrathecal synthesis of IgG in R-EAE and TMEV-IDD. Dot blot representing (**A**) the CSF levels of total IgG (mg/dL) measured by Luminex technology, (**B**) albumin CSF/serum quotients ($Q_{albumin}$), and (**C**) IgG indices ($Q_{Ig}/Q_{albumin}$) in R-EAE and TMEV-IDD mice as well as age-matched control mice (cR-EAE and cTMEV-IDD). Horizontal lines represent the median value for that group. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

DISCUSSION:

Quantitative methods for the evaluation of increased CSF protein concentrations are useful tools in the characterization of the physiological and pathological state of the CNS. However, beyond reliable quantification of CSF protein levels, the detection of CSF proteins requires an expression of results that discriminates between blood- and CNS-derived fractions in the CSF. However, to date, the commonly used protein quantification assays do not allow discrimination between the two protein components, even with the aid of high-throughput tools. Thus, specific corrections to protein measurements have been proposed in order to distinguish between proteins synthesized within the CNS compartment and proteins derived from the blood. Such corrections compensate for individual variability in both serum concentrations and barrier integrity. Overall, these corrections are based on calculations of a CSF/serum quotient ($Q_{protein}$), which reduces variability due to differences in the individual concentration of serum protein. Variation of the

$Q_{protein}$ related to individual differences in barrier function can be further lowered by relating the $Q_{protein}$ to a CSF/serum albumin quotient ($Q_{albumin}$). The combination of both corrections is generally known as protein index and is calculated as $Q_{protein}/Q_{albumin}$ ratio[4,6].

Albumin, synthesized and secreted by the liver, is the major plasma protein that circulates in the bloodstream. Because albumin is produced exclusively outside the CNS and its levels in CSF are low (~0.15 g/dL), increased CSF albumin levels indicate either damage to the BBI or blood contamination due to intrathecal hemorrhage or traumatic CSF collection. In any of these conditions, albumin transudates from serum into the CSF in proportion to its serum concentration. Therefore, in the absence of blood contamination, the $Q_{albumin}$ can be used as an indicator of barrier function[20]. Conversely, the $Q_{albumin}$ remains constant within normal ranges in humans and animals with an intact BBI[21]. A fundamental assumption for using this quotient[4,6] in intrathecal protein synthesis calculation is that the increased amount of CSF protein in the presence of a leaky barrier is proportional to the increase in CSF albumin concentration. This assumption has been experimentally confirmed in a study from 1977[4], in which authors monitored in MS patients the blood–CSF passage of radiolabeled IgG and albumin obtained from healthy human sera.

Similar to albumin, any protein in the blood can cross the BBI. When the barrier is intact, the $Q_{protein}$ is relatively constant. Unlike albumin, however, many proteins can also be synthesized within the CNS. As a consequence, an altered $Q_{protein}$ can result from a damaged barrier and/or increased intrathecal protein production. Nevertheless, when an elevated CSF protein concentration is due just to a compromised BBI, values for both $Q_{protein}$ and $Q_{albumin}$ are increased, compared to values for these same quotients in animals with an intact barrier. In contrast, when the barrier is intact, increased CSF protein concentrations are most likely due to increased intrathecal synthesis, and only the $Q_{protein}$ is ultimately increased.

The use of the protein index may be partially limited by five factors: 1) the large variability of CSF albumin concentration in healthy animals, 2) the different hydrodynamic radius of proteins, 3) the endogenous CNS expression of proteins, 4) different sampling techniques, and 5) the morphological differences of the BBI. The large variability of CSF albumin concentration in healthy animals results in considerable variability in the values for the final protein index. In humans, for example, the $Q_{albumin}$ is age-dependent since it increases with age[22]. A previous report also mentioned a sex-based difference in $Q_{albumin}$ in a healthy population[23]. Likewise, in mice albumin concentrations depend on age and mouse strain[24], e.g., the $Q_{albumin}$ for young, male, C57Bl/6 mice may be different from the $Q_{albumin}$ for old, female, DBA/1J mice. Therefore, standardized reference intervals for barrier integrity cannot be established across and within species, and appropriate thresholds have to be calculated based on the specific experimental conditions.

Another factor causing variability in normal indexes among proteins is their molecular size. The passage of serum proteins through the BBI depends on their molecular size, and the correlation between clearance rate and molecular weight (MW) is widely used to evaluate the permeability of the BBI. General protein structures range in size from tens to several thousand amino acids. Some proteins are of relatively small molecular size, such as chemokines, with a molecular weight

ranging between 8 and 20 kDa. Such a low MW favors crossing of the BBI, ultimately resulting in higher normal protein indexes. Differently, other proteins, like IgM, are very large (900−950 kDa), therefore showing very low indexes in normal conditions[6]. However, this is not always the case, since, despite a similar MW, some proteins permeate the barrier much better than other proteins, possibly because of a different shape. Thus, the diffusion coefficient of a protein, and hence the hydrodynamic radii calculated from it, depends on both size and shape of molecules[25]. The fundamental difference between the geometric and the hydrodynamic volume of a protein becomes most evident with large proteins above 150 kDa. The decreasing clearance rates of, for example, ceruloplasmin (132 kDa), IgG (150 kDa), and IgA (150 kDa) reflect the hydrodynamic heterogeneity of these three proteins, which have similar MW[25]. It is also possible that there is intrathecal production of proteins under normal circumstances. Some chemokines, e.g., CXCL10, are typically produced intrathecally, while others, e.g., CXCL13, are not[26,27]. This means that interpretation of protein indexes under most experimental conditions generally requires analysis of age-, sex-, and strain-matched untreated controls.

Protein levels in fluids can also be affected by different sampling techniques. While this may not be an issue for CSF collection as described here -there are no differences in CSF sampling between the described survival and non-survival procedures-, different blood collection methods may have an impact on the total serum protein amount. Some methods of blood collection yield arterial blood, others yield venous blood, while still others yield a mixture of both[14]. The sample obtained from survival retro-orbital bleeding is a mixture of venous blood and tissue fluid, whereas the terminal blood collection from the cardiac puncture can yield venous or arterial blood or a mixture of both[14]. In healthy animals, the total protein and albumin content of the arterial blood serum may be slightly higher than the same fractions of the venous blood serum[28]. This should be taken into consideration when survival and non-survival samples are compared.

Finally, an important feature to consider is the heterogeneous morphological structure of the BBI, which comprises at least two distinct barriers, the blood-brain barrier (BBB) located at the endothelium of the brain microvessels and the blood–CSF barrier (BCSF) located at the epithelium of the choroid plexuses[29]. Both barriers restrict and regulate the passage of molecules and cells between the peripheral and cerebrospinal compartments, although they do so by different mechanisms. While the BBB is a real physical barrier, characterized by a complex interplay among cells, the BCSF is more of a physiological barrier, which mostly depends on the CSF flow. Reductions in CSF production, release, and flow rate due to neurological conditions and/or trauma impair the BCSF function, thereby increasing the $Q_{albumin}$[9,30-32]. Therefore, $Q_{albumin}$ serves as a better marker of the BCSF permeability rather than the BBB or generally BBI permeability.

In summary, the calculation of a protein index is a relatively simple, and well-characterized method for discrimination between transudate and intrathecally produced proteins. The advantage of applying this formula to correct the general measurement of proteins in CSF samples is that it generates an objective variable to quantify the intrathecal synthesis of proteins and to measure the BBI, specifically BCSF, (dys)function. Given the robustness of this approach, the correction of the CSF protein amounts through the $Q_{albumin}$ and protein index provides a

baseline against which to assess (1) the pathophysiologic origin of any CSF protein and (2) the stability and functional significance of barrier integrity. Here it is presented a detailed protocol, from CSF and blood collection to the final calculations correcting the total CSF protein amount, which applies to mouse models for neurological diseases. However, the same protocol can be easily adapted to the study of CSF and intrathecal synthesis of proteins in any animal, including humans. $Q_{albumin}$ and IgG index are already commonly used in clinical practice for the diagnosis of inflammatory neurological diseases[6]. These same parameters have also been successfully used to evaluate a broad range of cytokines and chemokines in patients with inflammatory demyelinating diseases[26,33,34].

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The authors have nothing to disclose.

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Figure 1

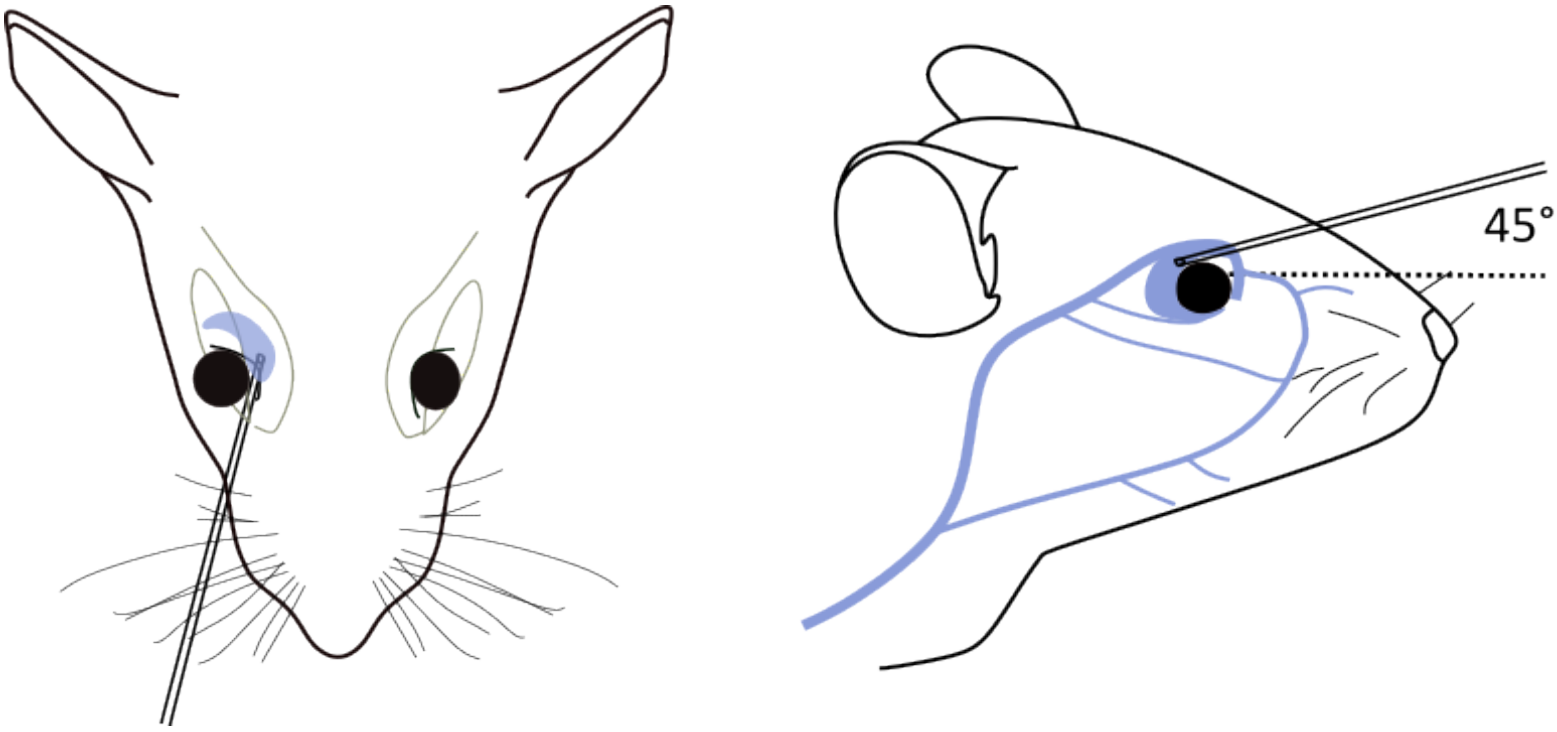


Figure 2

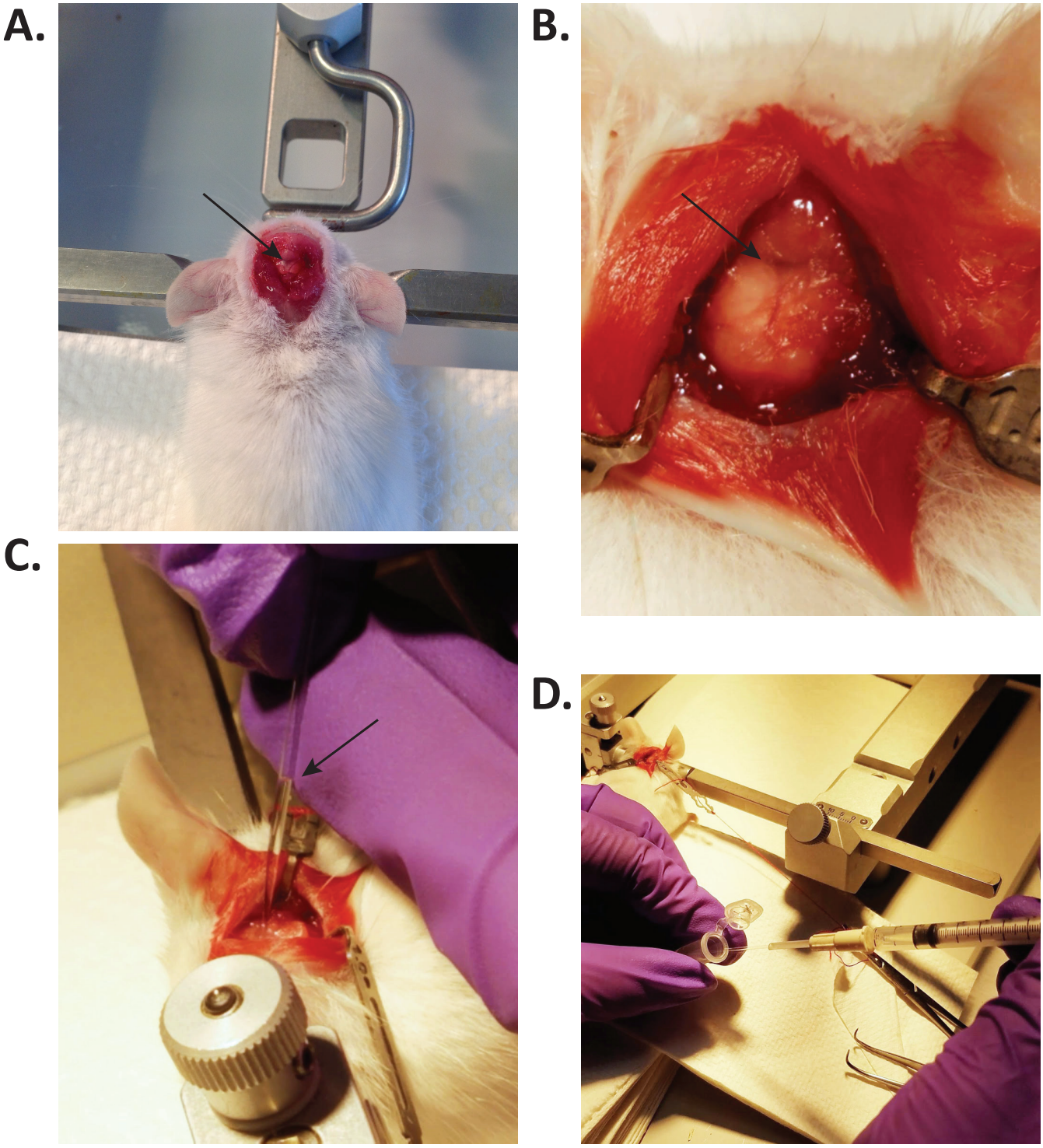
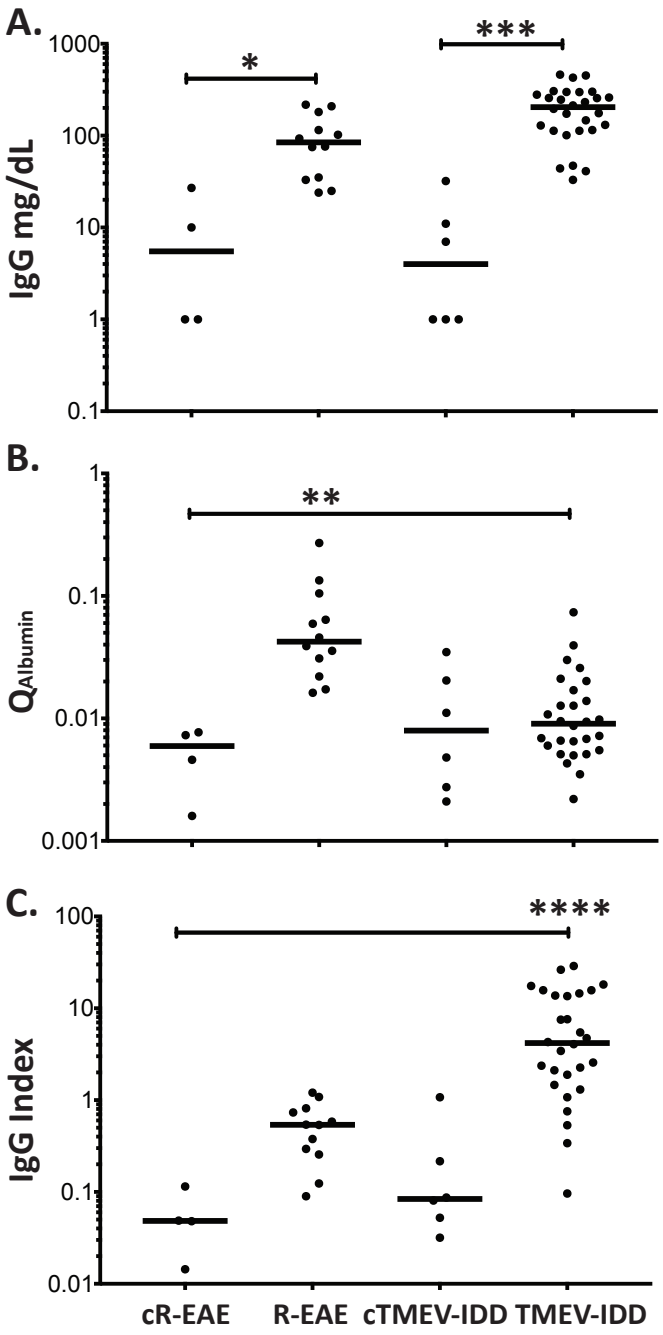


Figure 3



Name of Material/Equipment	Company	Catalog Number
1 mL insulin syringe	BD	329650
1 mL syringe	BD	329622
25 gauge needle	BD	305122
3 mL syringe	BD	309582
30 gauge insulin needle	BD	305106
Absorbent pads	Any suitable brand	
Acepromazine	Patterson Vet Supply Inc	
BioPlex Handheld Magnetic Washer	BioRad	171020100
BioPlex MAGPIX Multiplex Reader	BioRad	171015001
BioPlex Pro Flat Bottom Plates	BioRad	171025001
Biotinilated detection antibody	Any suitable source	
Bovine Serum Albumin (BSA)	Sigma	A4503
Buprenorphine hydrochloride	PAR Pharmaceutical	NDC 42023-179-05
Capillary Tubes	Sutter Instrument	B100-75-10
Centrifuge tube, 0.2 mL	VWR	20170-012
Centrifuge tube, 0.5 mL	VWR	87003-290
Centrifuge tube, 1.5 mL	VWR	87003-294
Chlorhexidine diacetate	Nolvasan	E004272
Disposable pipettes tips	Any suitable brand	
Ear bars	KOPF Instruments	1921 or 1922
Ethanol	Kopter	V1001
Freezer	VWR	VWR32086A
Gauze	Medline	NON25212
Heating pad	Sunbeam	
Induction Chamber	VETEQUIP	
Isoflurane	Patterson Vet Supply Inc	NDC 14043-704-06
Ketamine (KetaVed)	Patterson Vet Supply Inc	
MagPlex Microspheres (antibody-coupled)	BioRad	
Microplate Shaker	Southwest Scientific	SBT1500
Microretractors	Carfill Quality	ACD-010

Microsoft Office (Excel)	Microsoft	
MilliPlex MAP Mouse Immunoglobulin Isotyping Magnetic Bead Panel	EMD Millipore	MGAMMAG-300K
Mouse Albumin capture ELISA kit	Novus Biological	NBP2-60484
Multichannel pipette	Eppendorf	3125000060
Non-Sterile swabs	MediChoice	WOD1002
Oxygen	AIRGAS	OX USPEA
Pasteur Pippettes	Fisher	13-678-20A
PDS suture with disposable needle, 6-0 Prolen	Patterson Vet	8695G
PE-Streptavidin	BD Biosciences	554061
Pipetters	Eppendorf	Research seriers
Polyethylene tubing		
Refrigerated Centrifuge	Beckman Coulter	ALLEGRA X-12R
Scale	Uline	H2716
Scalpel	Feather	EF7281
Shaver	Harvard Apparatus	52-5204
Standard proteins	Any suitable source	
Stereotaxic instrument	KOPF Instruments	Model 900LS
Sterile 1 x PBS	Corning Cellgro	21-040-CV
Sterile saline	Baxter	0338-0048-02
Surgical Forceps Curved, 7 (2)	Fine Science Tools	11271-30
Surgical Scissors	Fine Science Tools	14094-11
Vaporizer + Flow meter	Moduflex Anhestesia Instruments	
Vortex	Fisher	02-215-414
Warming pad	Kent Scientific Corporation	RT-JR-20
Water Sonicator	Cole Parmer	EW-08895-01
Xylazine	Patterson Vet Supply Inc	

Comments/Description

Magnet

The antibody has to be directed against the species of the protein of interest.

OD: 1.0 mm, ID: 0.75 mm Borosilicate glass 10 cm; drawn over Bunsen to make ID smaller.

XL King Size SoftTouch, 4 Heat Settings with Auto-Off, Teal, 12-Inch x 24-Inch

Antibody-coupled magnetic bead

Blunt - 1 mm

Commercial kit for the quantification through Luminex of a panel of immunoglobulin isotypes and subclasses in mouse fluids.
Commercial kit for the quantification through ELISA of albumin in mouse fluids.

Need to be autoclaved for sterility

5 & 3/4"
P-3 Reverse Cutting, 18"

The best choice for a reference standard is a purified, known concentration of the protein of interest.
Standard Accessories

0.9 % Sodium Chloride Irrigation USP
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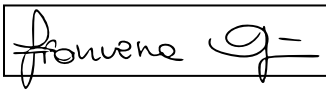
Signature:	
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Dear Sir/Madam

Thank you for the opportunity to submit a revised version of the manuscript mentioned above.

We appreciate the editor inputs. However, we feel like few crucial concepts need to be clarified regarding the protocol we are proposing for publication on JoVE. These should help the editor to better understand the crucial points of our protocol, in order to optimize the publication process.

1. The main point of the procedure is the final calculation of the albumin quotient and the protein index. Thus, it is imperative the final calculation steps are included in the highlighted content and therefore, in the video protocol.
2. One strength of our protocol is that it can be adapted to any quantification method, e.g., Luminex, ELISAs, or Simoa. By providing a detailed protocol for protein quantification by Luminex, we believe readers may feel confused and limited to a single quantification technology. We want to avoid this misunderstanding by keeping the protein quantification protocol more general, at least in the highlighted content.
3. Another strength of our protocol is that it can be applied to any protein. Regardless of the quantification method, different proteins require different and specific assay conditions. Therefore, we cannot provide unique quantification conditions, specifically sample dilutions in CSF and serum or detection antibodies, with this protocol.
4. Representative results were obtained using commercial quantification kits. E.g., the MilliPlex MAP Mouse Immunoglobulin Isotyping Magnetic Bead Panel from Millipore, was used to quantify IgG levels in mouse CSF and serum specimens. We do not want (and we are not allowed) to publish a protocol based on a commercial kit. Therefore, we are providing a more general protocol, without referring to IgG, which is not the one we used with the kit.
5. MagPlex Microspheres (antibody-coupled) and MilliPlex MAP Mouse Immunoglobulin Isotyping Magnetic Bead Panel are trade names for the magnetic beads and the Luminex quantification kit respectively. As instructed by the editor, trade names have been deleted by the text.

We have revised the manuscript per the editor suggestions, although we do not feel like some of the changes, specifically those regarding the quantification method, actually improve the manuscript. As requested, revisions to the body of the manuscript were emphasized with track changes. We hope that the revised manuscript along with our explanations above will now be acceptable for publication on JoVE.

Sincerely,
Francesca Gilli, PhD