Video Article

Intravascular Perfusion of Carbon Black Ink Allows Reliable Visualization of Cerebral Vessels

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Abstract

The anatomical structure of cerebral vessels is a key determinant for brain hemodynamics as well as the severity of injury following ischemic insults. The cerebral vasculature dynamically responds to various pathophysiological states and it exhibits considerable differences between strains and under conditions of genetic manipulations. Essentially, a reliable technique for intracranial vessel staining is essential in order to study the pathogenesis of ischemic stroke. Until recently, a set of different techniques has been employed to visualize the cerebral vasculature including injection of low viscosity resin, araldite F, gelatin mixed with various dyes[1] (*i.e.* carmine red, India ink) or latex with[2] or without[3] carbon black. Perfusion of white latex compound through the ascending aorta has been first reported by Coyle and Jokelainen[3]. Maeda *et al.*[2] have modified the protocol by adding carbon black ink to the latex compound for improved contrast visualization of the vessels after saline perfusion of the brain. However, inefficient perfusion and inadequate filling of the vessels are frequently experienced due to high viscosity of the latex compound[4]. Therefore, we have described a simple and cost-effective technique using a mixture of two commercially available carbon black inks (CB1 and CB2) to visualize the cerebral vasculature in a reproducible manner[5]. We have shown that perfusion with CB1+CB2 in mice results in staining of significantly smaller cerebral vessels at a higher density in comparison to latex perfusion[5]. Here, we describe our protocol to identify the anastomotic points between the anterior (ACA) and middle cerebral arteries (MCA) to study vessel variations in mice with different genetic backgrounds. Finally, we demonstrate the feasibility of our technique in a transient focal cerebral ischemia model in mice by combining CB1+CB2-mediated vessel staining with TTC staining in various degrees of ischemic injuries.

Video Link

The video component of this article can be found at http://www.jove.com/video/4374/

Protocol

1. Animals

1. Experiments were carried out according to the NIH guidelines for the care and use of laboratory animals and approved by local authorities. For all experiments, C57Bl6/J wild type mice, ApolipoproteinE$^{-/-}$ (ApoE KO) and SV129 mice (12-16 weeks old, 26-30 g body weight, 5-6 animals per experimental group) were used.

2. Staining of Cerebral Vessels with Colored Latex

1. Prepare a mixture of 25 µl carbon black ink (Herlitz, Germany) with 0.5 ml of latex compound (Pebeo, France) at a 1:20 ratio in an EP tube and warm up the mixture at 37 °C in a water bath. Collect the mixture in a 2 ml syringe with a needle of 28-30G before anaesthetizing the animal.
2. Dissolve 50 mg of papavarine hydrochloride powder into 1 ml of sterile normal saline. Collect the solution in an insulin syringe. Break off the needle from another sterile insulin syringe. Place this needle at the end of a 20 cm long PE10 tube. Now attach this PE10 tube on the needle of the insulin syringe containing papavarine hydrochloride solution to be injected through the femoral vein to ensure vasodilatation and proper filling of vessels.
3. Prepare another two insulin syringes each containing 1 ml of saline. Insulin syringes allow smooth delivery of the injected fluid at a precise location. However, due to high viscosity of latex, alternative 1 ml syringes with removable wider needles should be used.
4. Take all the syringes and sterilized dissecting instruments close to the operating stage. Spread a surgical draper sheet around the operation stage. The operation stage should be sufficiently illuminated and an operating microscope may be used if necessary.
5. Now measure the body weight of a C57Bl6/J mouse and then induce anesthesia with 5% vaporized isoflurane in 70% N_2O and 30% O_2. Continue anesthesia with 1% of vaporized isoflurane. After proper positioning of the mouse on its back and fixation of the limbs, assess the

depth of anesthesia. Then cut the skin over the left femoral vein and locate the vein. Insert the sharp tip of the needle placed on PE10 tube and slowly inject papavarine hydrochloride solution at a rate of 100 µl per min (50 mg/kg body weight).
6. Following the injection, cut the abdominal cavity and pierce the diaphragm. Cut the ribs to expose the heart without damaging or bruising it. Clip the descending thoracic aorta with an artery forceps. Make a sharp cut over the right atrium to allow the venous blood drain out. Inject 2 ml of saline into the left ventricle, followed by injecting the colored latex manually with slight pressure over 18-20 sec. Use the pre-filled syringes one after another. Avoid injecting any bubbles.
7. Following injection of latex, leave the animal for 5 min. Decapitate the animal and carefully remove the whole brain. Take good care not to injure the vessel architecture and cortex while removing the skull bone and meninges. Place the brain in a 60 mm cell culture dish containing 6-8 ml of 4% PFA to take a photo. Place a measuring ruler under the transparent dish. Take photos at a 25x magnification of the dorsal and the ventral surface of the brain with a camera attached to the operating microscope. Photos should be taken at 90° angle between the Petri dish and the microscope objective in order to quantify the original distance.
8. Repeat the procedure in another 4-5 animals.

3. Staining of Cerebral Vessels with Mixture of Carbon Black Inks

1. To compare the outcome of latex based cerebral vascular staining with our protocol, prepare a mixture of 100 µl of Herlitz Stempelfarbe ink (CB1) with 900 µl of Pelican Scribtol Schwarz ink (CB2) in a 1.5 ml EP tube. Prepare two EP tubes to make a total volume of 2 ml mixture of CB1 and CB2 at a 1:9 ratio. Collect the mixture in two separate insulin syringes (1 ml each). Cap the syringes and place them in a water bath to warm up to 37 °C. Collect 2 ml of saline in another two insulin syringes, and place them in a water bath as well.
2. Anesthetize the animal and repeat the same procedure as described above except for injection of papavarine hydrochloride. Following injection of 2 ml salaine into the left ventricle, inject a 2 ml mixture of carbon black inks instead of the colored latex. Perform the injections by hand with slight pressure over 18-20 sec per ml. Clip the descending thoracic aorta prior to the injections.
3. Sacrifice the animal, remove the brain and take photos.
4. For long-term preservation of the brain, put the brain in 4% PFA overnight followed by incubation with sucrose with gradually increasing concentration until the floating brain submerges totally (5% followed by 15% and then 30%). Brains of the animals perfused with the colored latex can also be preserved in the same way.
5. Perform the procedure in another 4-5 animals. To compare the difference in cerebral vascular anatomy due to different genetic background or strains, repeat the protocol in equal number of ApoE KO and SV129 mice, respectively.
6. To study the vascular anatomy under ischemic conditions, induce transient focal cerebral ischemia for 45 min or 90 min according to a standard protocol of intraluminal occlusion of the MCA under deep anesthesia (a detailed description of the surgery is beyond the scope of this article; the reader is referred to Doeppner et al., 2010). At the end of the reperfusion period planned (1 day or 5 days), perform the vascular staining with CB1+CB2 inks only and stain the whole brain with 2% TTC solution at 37 °C for 5-10 min to identify the infarct volume. TTC is reduced to red colored formazan by the mitochondrial enzymes (particularly succinate dehydrogenase). After TTC staining, the metabolically active tissue stains deep red while the infarcted tissue remains unstained and appears white due to dysfunctional and denatured mitochondrial enzyme. Collect images in the same way as described above.

4. Study of Cerebral Vascular Territories

1. To analyze the gross anatomy of cerebral vessels, images of the dorsal surface of the brain either stained with colored latex or CB1+CB2 inks can be analyzed with ImageJ software (a Java based public domain software used for image processing and analysis). Brains perfused with colored latex may show variable degrees of vessel staining located on the dorsal surface, while CB1+CB2 perfusion will stain all the vessels on both the ventral and dorsal surfaces.
2. Open an image file with ImageJ software. Identify all the anastomotic points between the ACA and the MCA. An anastomotic point is defined as the site where the vessel diameter is the narrowest or the half distance between the nearest branching points of the ACA and the MCA branches, respectively[2]. Draw an imaginary line (anastomotic line) by marking the anastomotic points with the help of drawing segmented line tool and the plugin "Dotted Line".
3. Set the scale in mm. Measure the distance between the anastomotic line and the midline at 4 mm caudal to the frontal pole of the brain using straight line drawing tool and "measure" tool. Do the same at 6 mm caudally from the frontal pole. Analyze 3-4 images per animal and calculate the mean value. Compare the values among different groups of animals to identify the variation of cerebrovascular anatomy. In C57Bl6/J animals, the anastomotic line between the ACA and the MCA can be traced closer to the midline at 4 mm than at 6 mm caudally from the frontal pole. ApoE KO mice will present no significant difference in this respect. On the contrary, in SV129 mice the anastomotic line will lie further and parallel to the midline both at 4 mm and 6 mm caudally from the frontal pole.
4. To analyze the vascular anatomy in ischemic conditions, perform the same calculations mentioned above. Identify the infarction border as the red and white colored tissue margin representing metabolically active tissue and necrotic unstained tissue, respectively. Measure the distance of the infarction border from the midline at 4 mm and at 6 mm caudal from the frontal pole of the brain. Collect the mean value from 3-4 images per animal. Compare the results between different ischemia and reperfusion periods.

Representative Results

The protocol described here overcomes the technical limitations of conventional latex based visualization of rodent cerebral vasculature. **Figure 1A** shows that following perfusion of the colored latex, only the large vessels on the ventral surface are stained, leaving the entire dorsal surface unstained. The outcome is also highly variable. Only one animal out of six shows partial staining of the ACA and the MCA visible on the dorsal surface of the brain (data not shown). Conversely, CB1+CB2 perfusion results in sufficient filling of both small and large vessels in an equal manner (**Figure 1B**). The staining is stable until 7 days without any change of quality. Using ImageJ programme, we have quantified the distance of the anastomotic points between the ACA and the MCA from the midline in wild type C57Bl6/J mice (**Figure 1C**). We compared these values with ApoE KO mice to observe whether or not the genetic modification had any effect on the regional vascular anatomy (**Figure 1D**). Although knocking out of ApoE did not affect the anatomical structure of the cerebral vessels in C57Bl6/J mice, a significant difference was noticed

between C57bl/6J and SV129 strains (**Figure 1E, F**). Thus, we are able to show the feasibility of this staining protocol to evaluate the anatomical differences of the cerebral vasculature due to genetic or strain differences in mice.

In experimental rodent stroke models, TTC staining is widely used to observe the infarction volume. Simultaneous visualization of cerebral vessels and the infarction volume give us the opportunity to analyze morphological changes following ischemia-reperfusion. Therefore, we have verified whether or not CB1+CB2-mediated vascular staining was stable with TTC staining in various degrees of ischemic-reperfusion injuries (**Figure 2A-H**). We have further analyzed the anastomotic points between the ACA and the MCA after 45 min or 90 min of ischemia matched with either 1 day or 5 days reperfusion periods (**Figure I, J**). As expected, the mean distance between the midline and the anastomotic points showed no significant difference among the groups (**Figure 2I, J**) and was in the same range with non-ischemic animals (**Figure 1F**). However, the distance from the midline to the infarction border differed significantly, reflecting the increased infarction volume with higher degree of ischemia-reperfusion injury (**Figure 2K, L**). These data show that CB1+CB2 staining can be reproduced in the animals subjected to various degrees of ischemia-reperfusion injury and the stability of the staining when combined with TTC staining.

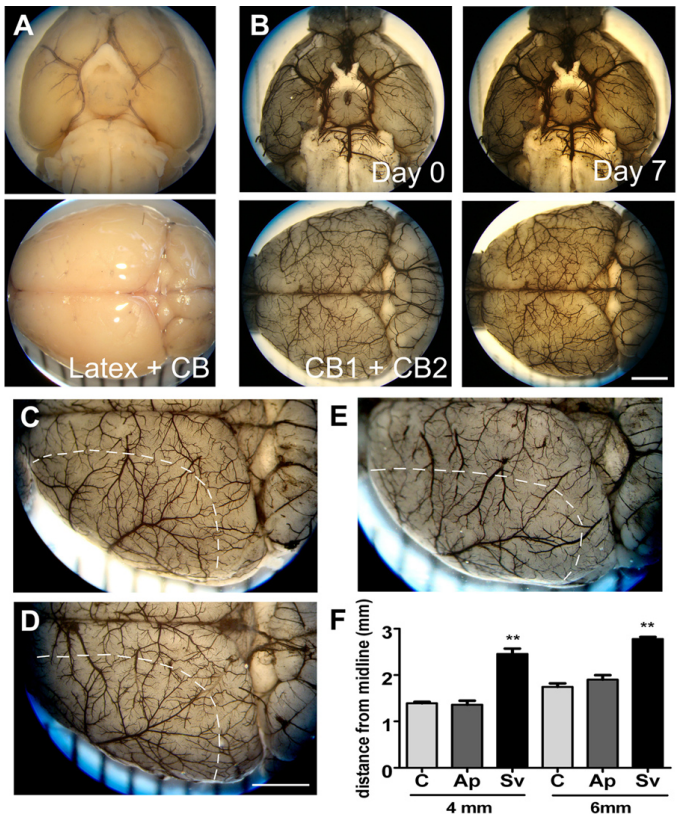

Figure 1. Observation of the cerebral vascular anatomy in mice with genetic and strain differences. Figure 1A shows that intravascular perfusion of colored latex only stains large vessels on the ventral surface, while the dorsal surface remains unstained. Conversely, CB1+CB2 perfusion results in permanent staining of both small and large vessels on the ventral and the dorsal surfaces of the brain (**Figure 1B**). Quantification of the distance of the anastomotic points between the anterior cerebral artery (ACA) and the middle cerebral artery (MCA) from the midline in wild type C57Bl6/J mice (**Figure 1C**) show no difference with their genetically mutant ApoE KO counterparts (**Figure 1D**). However, significant difference is noticed between C57Bl6/J and SV129 strains (Figure 1E, F). Scale bar = 2mm. * Significantly different from both C57BL6/J wild type and ApoE KO mice at 4mm, $p<0.05$, and ** at 6 mm, $p<0.01$.

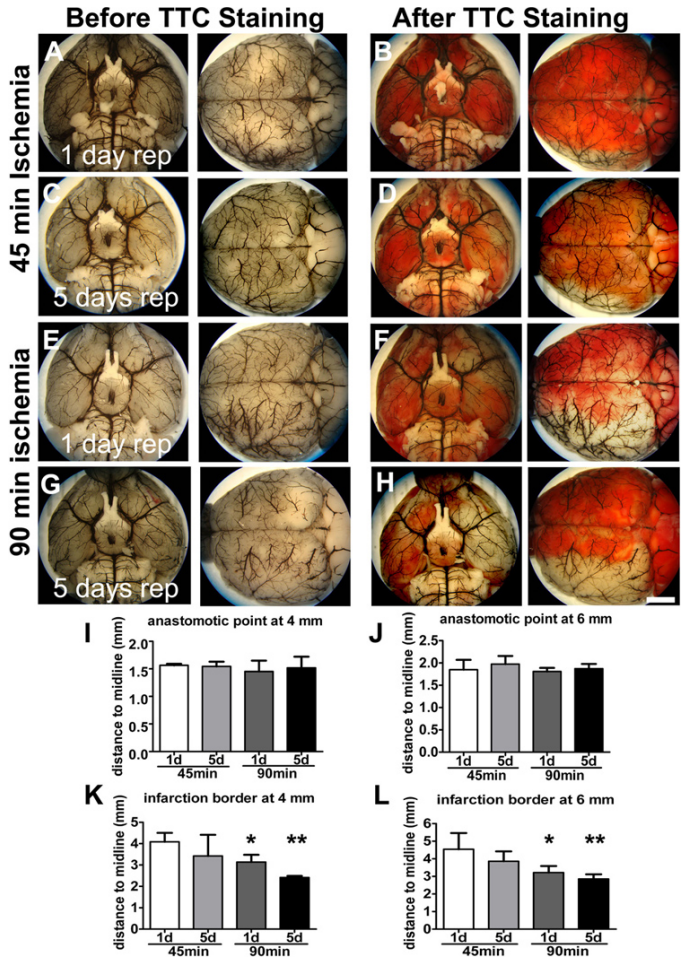

Figure 2. Analysis of vascular anatomy in ischemic brains. Figure 2A-H show images of the ventral and dorsal surfaces of the brains subjected to 45 min or 90 min ischemia followed by 1 day or 5 days reperfusion time. The left panel shows CB1+CB2 mediated vascular staining, whereas the right panel shows combination of both the vascular staining and TTC staining, indicating the stability of the vascular staining in various degrees of ischemic-reperfusion injuries (**Figure 2A-H**). Analysis of the anastomotic points between the anterior cerebral artery (ACA) and the middle cerebral artery (MCA) after 45 min or 90 min ischemia matched with either 1 day or 5 days reperfusion period depicts no significant difference among the groups (**Figure 2I, J**). However, significant difference can be noted in the distance from the midline to the infarction border, indicating the increase of infarction volume with higher degree of ischemia-reperfusion injury (**Figure 2K, L**). Scale bar = 2 mm. * Significantly different from the 45 min ischemia with 1 day reperfusion, $p<0.05$, and ** 5 days reperfusion, $p<0.01$. Click here to view larger figure.

Discussion

Perfusion of CB1+CB2 by manual injection can be carried out successfully by without intensive training as it does not involve any specific device to imply certain pressure[2,3]. The heterogeneity of perfusion outcomes in our protocol is also negligible. Only 1 animal out of 16 non-ischemic animals and 3 out of 20 ischemic animals have showed incomplete perfusion. In these cases, incorporation of bubbles during saline perfusion leading to occlusion of vessels was most likely the reason of the unsuccessful outcome.

Although perfusion of latex has originally been carried out through the ascending aorta [2,3], alternative routes of perfusion are also reported including the left ventricle [7,8] or the common carotid artery[9]. We found no significant difference in the outcomes following perfusion through either the ascending aorta or the left ventricle (unpublished observations). As such, the latter was chosen due to its easier accessibility.

Before selecting the specific carbon black inks mentioned in this study, we have evaluated the outcomes of intravascular perfusion with a range of commercially available carbon black inks. CB1 and CB2 are selected for their perfusion efficiency and stability of staining. CB1 has a very low viscosity (1.1 mPa/sec) containing 2.1% carbon black while CB2 - originally manufactured as a calligraphy ink - contains 2.5% carbon black with a higher viscosity (10-50 mPa/s). Individual application of these inks has been found to be insufficient regarding both efficiency and stability of staining. Consequently, we have combined the inks and titrated the combination ratios. Perfusion with CB1:CB2 at a 1:9 ratio results in permanent staining of both large and small vessels. Therefore, arterial anastomotic points can be easily traced to define the junction between two vascular territories, *i.e.* the ACA and the MCA. These findings are consistent with previous reports [2,10,11].

ApoE KO mice present 5-8 times higher plasma cholesterol level and are highly susceptible to developing atherosclerosis when put on cholesterol rich diet[12]. Whether the genetic modification also affects the anatomy of cerebral vessels has not been reported yet. On the other hand, SV129 mice are already reported to present larger infarction volume in comparison to C57Bl6/J mice due to their different vascular anatomy[2]. We verified our protocol in these three groups of animals to compare the difference in vascular anatomy.

To validate the staining protocol in ischemic brains, we involved transient focal cerebral ischemia to include effects of reperfusion in contrast to previous studies, which mainly focused on permanent occlusion of the MCA [2-4, 10,13]. As expected, we did not observe any significant differences in the pattern of the line of anastomosis after different ischemic and reperfusion periods in C57BL6/J mice. No changes in vessel diameters between the ischemic and non-ischemic hemispheres were observed, presumably, such changes occur at longer reperfusion times, *i.e.* 2-3 weeks post-stroke [14]. In conclusion, the protocol demonstrated by us is a less-complicated, cost-effective technique, which can be easily reproduced in comparison to conventional latex based perfusion to visualize cerebral vasculature. It may be considered as an effective tool to analyze the gross anatomy of cerebral vessels and vascular territories in different physiological and pathological conditions. By combining with TTC staining this technique provides a new opportunity to identify the extent of an ischemic insult in the context of vascular supply zones in the brain.

Disclosures

No conflicts of interest declared.

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