Video Article

Trypsin Digest Protocol to Analyze the Retinal Vasculature of a Mouse Model

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Abstract

Trypsin digest is the gold standard method to analyze the retinal vasculature [1-5]. It allows visualization of the entire network of complex three-dimensional retinal blood vessels and capillaries by creating a two-dimensional flat-mount of the interconnected vascular channels after digestion of the non-vascular components of the retina. This allows one to study various pathologic vascular changes, such as microaneurysms, capillary degeneration, and abnormal endothelial to pericyte ratios. However, the method is technically challenging, especially in mice, which have become the most widely available animal model to study the retina because of the ease of genetic manipulations [6,7]. In the mouse eye, it is particularly difficult to completely remove the non-vascular components while maintaining the overall architecture of the retinal blood vessels. To date, there is a dearth of literature that describes the trypsin digest technique in detail in the mouse. This manuscript provides a detailed step-by-step methodology of the trypsin digest in mouse retina, while also providing tips on troubleshooting difficult steps.

Video Link

The video component of this article can be found at http://www.jove.com/video/50489/

Introduction

Visualizing the vasculature of the retina is an extremely important approach to dissect the mechanisms of various eye diseases such as diabetic retinopathy. It allows one to assess the earliest vascular abnormalities, including microaneurysms, capillary degeneration, and pericyte loss [8,9]. To date, there have been several techniques developed to analyze the retinal vasculature. Perfusion of various dyes has been used to highlight the vessels, but all have shared similar limitations. Injection rarely highlights the entire retinal vasculature unless given at a high pressure, which risks rupturing and damaging the vessels [1]. Immunostaining of vascular endothelium with fluorophore-labeled G isolectin B4 (Alexa Fluor 594 conjugated; I21413; Invitrogen; 1:100 dilution) and retinal flat mounts can highlight the overall architecture of the vessels, but without detailed visualization of capillaries, basement membranes and pericytes. It was noted by Friedenwald that vessels can be highlighted by staining flat-mounts of retina with periodic acid-Schiff (PAS) or hematoxylin and eosin (H&E) staining [10]. However, staining was non-specific to the vessels and highlighted the non-vascular tissue as well, making it difficult to differentiate the vessels. In the 1960s, Cogan and Kuwabara developed the trypsin digest technique that made it easier to visualize the retinal vasculature by digesting the nonvascular components of the retina [1]. Since that time, the trypsin digest has become the gold standard method in analyzing the vasculature of the retina [2-5]. However, it is important to note that other alternative techniques to isolate the vasculature have been described. The use of osmotic lysis has been used to isolate the vasculature and allow biochemical studies of the tissue [11,12], but the procedure has not been used as a primary method for anatomical study. The tissue print method has been used to isolate large segments of microvasculature and allows the ability to study the electrotonic architecture of the vasculature [13]. In theory, this technique could also be used to study anatomical changes, as the quality of the vessels is high. However, it is only able to isolate segments of the entire vasculature network. Although these methods cannot replace trypsin digest, it is important to note that they have different advantages and shortcomings and are complementary in this regard.

The trypsin digest method is technically challenging and is difficult to perform consistently [6,7]. Furthermore, it has been noted that trypsin digest is especially difficult on a mouse model, particularly if one desires to preserve the overall vascular architecture of the retina [6,7]. Challenges include (1) over-digestion of the retina that causes loss of both the vasculature as well as non-vascular tissue, (2) under-digestion, requiring extensive mechanical dissection which could in turn lead to damage of the vessel bed, (3) poor separation of the non-vascular tissue from the vasculature leading to non-specific staining. Several manuscripts have highlighted these challenges, but none have provided a detailed and consistent protocol to overcome them [14,15]. This manuscript will introduce a step-by-step methodology detailing the technique to perform the trypsin digest on mouse and rat retinas with specific tips on handling the particularly difficult steps. A schematic overview is shown in **Figure 1**.

Protocol

1. Retinal Preparation

1. Enucleate the mouse eye. Using one hand, open the eye lids so that the eye is visible. With the other hand, using a curved forceps (curved facing upwards), apply pressure on superior and inferior aspects of the orbit until the globe protrudes. Gently close forceps at the posterior aspect of the eye and lift in a continuous motion to enucleate the eye.
2. Fix eye with 10% neutral buffered formalin for at least 24 hr.
3. Place eye in PBS solution in a small Petri dish.
4. Dissect out retina carefully under a microscope, taking care to avoid inducing large tears. Make an initial cut in the cornea with dissection scissors. Then, while holding the cut area with straight forceps, use scissors to cut along the border of the cornea and sclera removing the cornea. Using fine curved and straight forceps, carefully peel the sclera and choroid away from the retina towards the optic nerve. This step requires patience as quickly freeing the retina can lead to tears. Remove the lens and any excess iris and debris from the retina [16].

2. Water Washes

1. Place retina in a 24-well dish and cover with water (approximately 500 µl, filtered with at least a 45 µm filter).
2. Change water every 30 min-1 hr, at least 4-5 times.
3. Leave overnight in water with gentle shaking at room temperature.

Note: *The washing steps are very important as they help separate the neural retinal layers from the blood vessels.*

3. Trypsin Digest

1. Remove water and incubate retina in a solution of 3% trypsin (Difco 1:250) in 0.1 M Tris buffer (pH 7.8) at 37 °C with **gentle** to no shaking. Trypsin digest is completed when the tissue begins showing signs of disintegration (approximately 1.5 hr).

   Note: *Avoid rapid shaking as this can damage the vasculature.*

2. Carefully, pipette out trypsin into a separate well using a microscope to avoid touching the retina. This excess trypsin can be used later, when manipulating the vessels under the microscope, in step 4.3.

4. Separating the Vasculature

1. Add filtered water to the retina. Then, attempt to peel off the internal limiting membrane with forceps, using scissors if necessary. Shake with mild agitation for 5 min. Carefully, pipette out the water under the microscope to avoid damaging the retina.
2. Repeat water washes (for 5 min each) to help free the network of vessels from the adherent retinal tissue. Water washes are completed when there is little to no debris remaining in the water after wash.
3. Careful manipulations under a dissection microscope are now needed to further free up the network of vessels. Below are several useful techniques, which may be used in sequence or individually to achieve the desired outcome based on technical preference:
   a. Using a 200 µl pipette, carefully pipette water up and down adjacent to vessels, blowing water at vessels, causing gentle agitation.
   b. Using 200 µl pipette, pipette the trypsin up and down to help coat walls of the pipette. Then, CAREFULLY pipette the entire vessel network up and down **once** to help break down non-vascular tissue.

      Note: *Centering the pipette tip at the optic nerve rather than the edges of the vascular network can also prevent the vasculature from sticking to the tip.*

   c. Gently lift vasculature with fine forceps out of water to dislodge non-vascular components.
   d. If the above steps do not work, add trypsin to the retina to help break down the tissue, and incubate at 37 °C for several minutes. Then, remove trypsin and repeat steps 4.1-4.3.

Note: *Make sure to coat all instruments that will come into contact with the vessels in trypsin (e.g. pipette tip, forceps). This will help avoid adhesion of vasculature to the equipment.*

5. Visualizing the Vasculature

1. Place a drop of water onto a clean slide. Using a 200 µl pipette or forceps, transfer the digested vessels into the water. Make sure the vascular flat-mount is unfolded. *The surface tension of the water helps to unfold the now diaphanous vascular network.* If the tissue does not unfold, add additional water and gently shake the slide to facilitate unfolding.
2. Remove excess water very slowly by pipetting or using a paper towel.
3. Allow slides to dry completely.
4. Stain slides via appropriate protocol with either PAS/hematoxylin or H&E stain.

   Note: *PAS/hematoxylin is useful for identifying vessel wall and cellular nuclei; H&E is useful for demonstration of RBC as well.*

5. Dehydrate and mount (Permount mounting medium).

Representative Results

The final product of a successful procedure is a flat-mount of the entire network of the mouse retinal vasculature, with the architecture maintained, stained with either PAS/hematoxylin or H&E, as shown in **Figures 2-4**. Clear differentiation of endothelial cells and pericytes can be seen as shown in **Figure 3**. In the retina, the nuclei of endothelial cells are oval or elongated and lie entirely within the vessel wall. Pericyte nuclei are small, spherical, stain densely and generally have a protuberant position along the capillary wall.

Trypsin digest is a common procedure to analyze vascular pathology in diabetic animal models. Pathologic vascular lesions such as microaneurysms, pericyte ghosts (evidence of pericyte loss), acellular capillaries and capillary degeneration have been described. **Figure 4** shows an example of capillary degeneration seen in a db/db mouse, a model for type II diabetes. Although the procedure was optimized for mouse retinas, similar results have also been obtained in rat retinas as shown in **Figure 5**. It's important to note that rat retinas are much larger, and a 12-well plate was used instead of a 24-well plate. The vasculature network of the rat retina is also less fragile than the mouse counterpart.

It is extremely crucial to remove as much of the non-vascular tissue as possible. Any remnants will lead to non-specific staining and lead to poor visualization of the vasculature. **Figure 6** represents an example of unsuccessful removal of the entire non-vascular tissue in step 4. Likewise, it is also important to have the retina be unfolded when mounting so as to improve visualization of the vasculature. **Figure 7** represents unsuccessful unfolding of the vascular flat-mount in step 5.1.

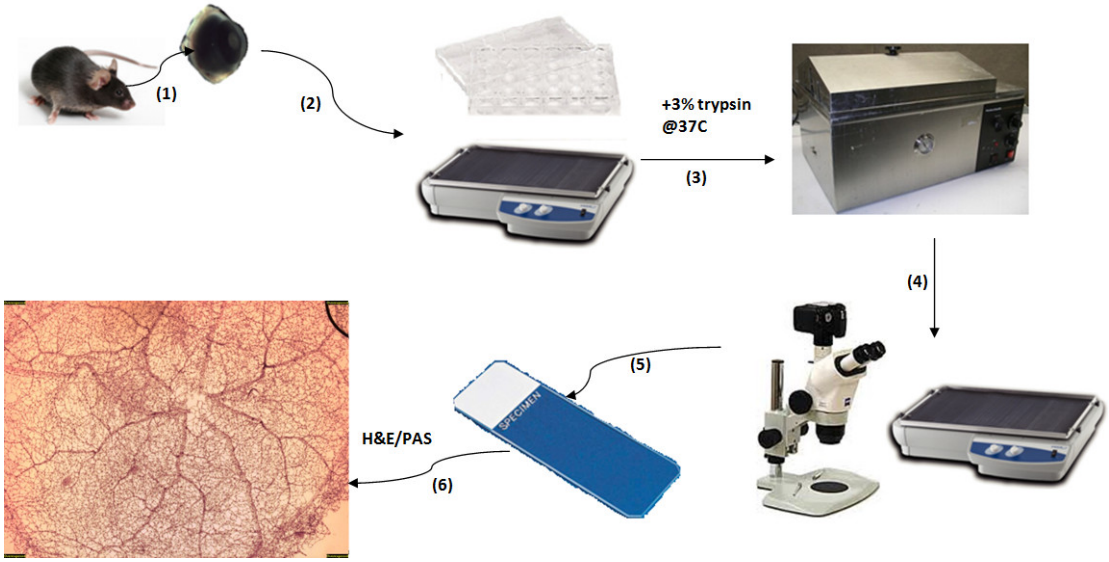

Figure 1. Schematic of Trypsin Digest Protocol. (1) *Retinal Preparation:* isolate retina from eye; (2) *Water washes*: Wash in filtered water overnight; (3) *Trypsin Digest:* Incubate in 3% trypsin at 37 °C for 1-1.5 hr; (4) *Separating the vasculature:* isolate vasculature via series of water washes and dissection under microscope; (5) Move vasculature to slide and let dry; (6) *Visualizing the vasculature:* stain slide with PAS or H&E, dehydrate and mount. Click here to view larger figure.

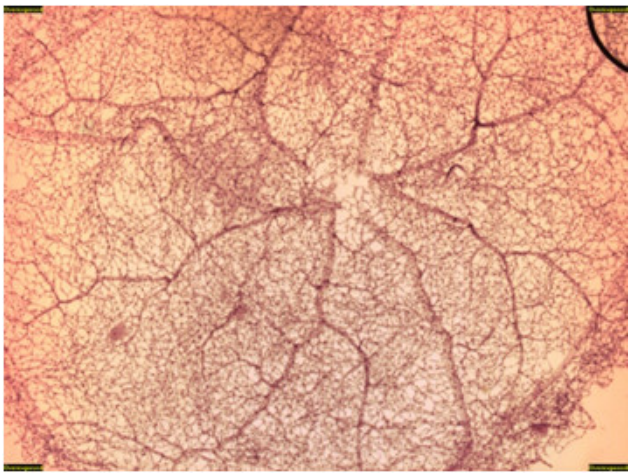

Figure 2. Control db/m mouse trypsin digest (H&E, 20x). This figure is an overview of the retinal vascular network showing normal vascular architecture.

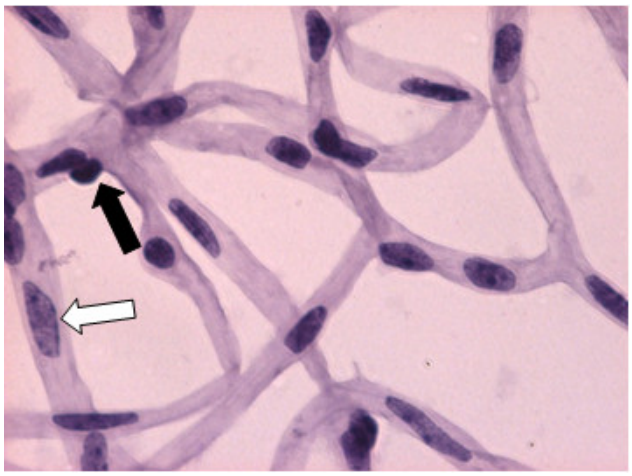

Figure 3. Control db/m mouse trypsin digest (H&E, 600x). This is a magnified view of the retina in **Figure 2** showing normal vascular architecture. Endothelial cell (white arrow) and pericyte (black arrow) are highlighted.

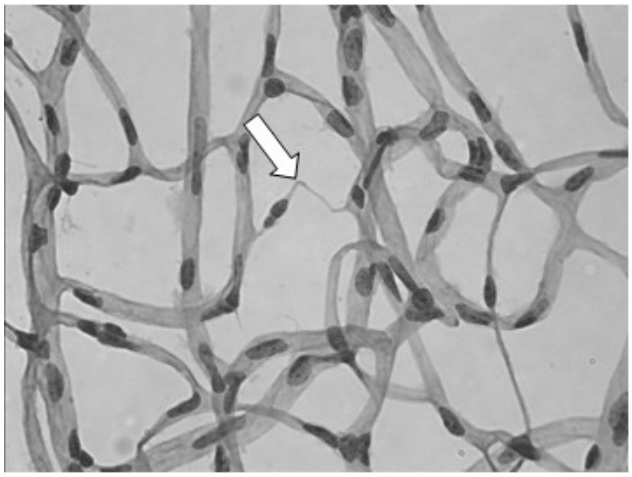

Figure 4. Diabetic db/db mouse trypsin digest (H&E, 500x). By 52 weeks, db/db mice begin to develop vascular pathology such as capillary degeneration (white arrow).

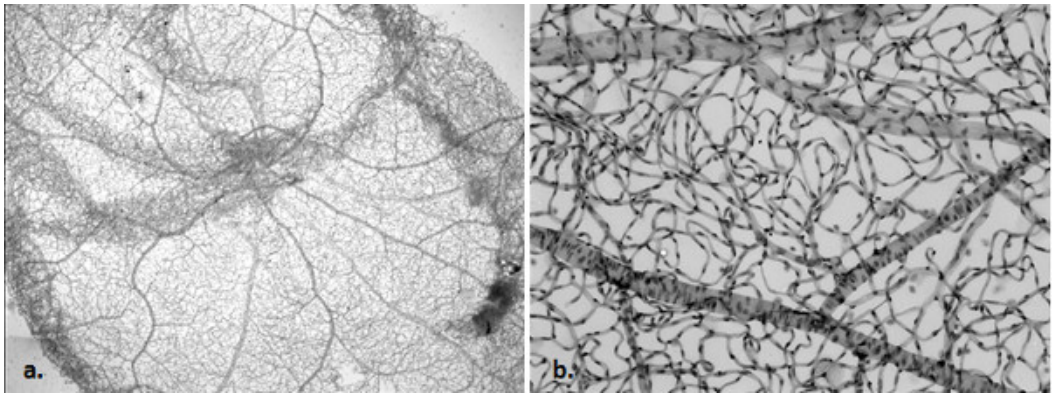

Figure 5. Normal rat retina trypsin digest, (PAS, 20x [a], 100x [b]). An overview and magnified image of a normal rat vascular network.

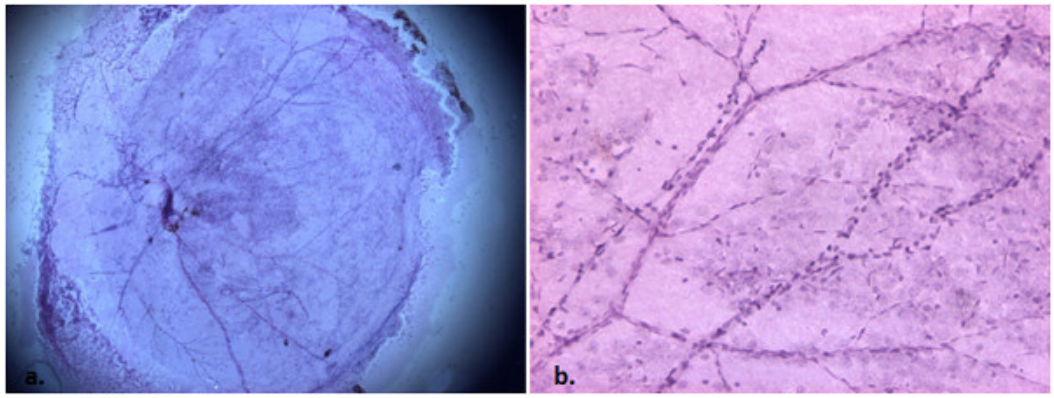

Figure 6. Control C57/Bl6 mouse trypsin digest, non-vascular tissue intact (H&E, 20x [a], 100x [b]). With poor removal of the non-vascular tissue, there is non-specific staining impairing visualization of vessels.

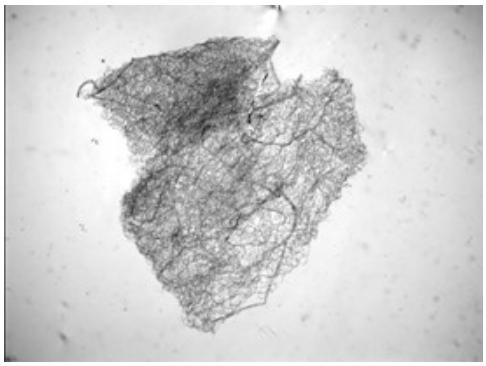

Figure 7. Control db/m mouse trypsin digest, poor mounting (H&E, 20x). If the retina is not adequately unfolded, there is inadequate visualization of the vascular network.

Discussion

Trypsin digest is a standard method to assess the vasculature of the retina. Unfortunately, it is technically challenging and can result in high rate of specimen loss if not performed correctly. Furthermore, the procedure is especially difficult in mice, which can limit the application of this technique in the commonly used genetic animal models of eye diseases. This paper provides guidance on how to perform the procedure effectively and consistently in mouse eyes.

There are several critical steps in the protocol. First, it is crucial that the retina be dissected out carefully to avoid any large tears, to ensure that the vascular network remains intact. Second, washing the retina in filtered water overnight is important as it helps separate the neuroretinal layers from the blood vessels, which facilitates an easier separation after trypsin digest. One modification that could expedite the process would be to place the retina in 1% triton X-100 mixed in filtered water for 1-2 hr. Then, trypsin digest can be performed the same day after a series of water washes to remove the triton X.

It is important that the retinal tissues be monitored constantly during the trypsin digest in order to avoid inadvertent dissolution of vasculature tissue. Based on our experience, around 1-1.5 hr is sufficient to accomplish digestion of the neural tissue, while preserving the vasculature intact. Less time is needed for younger mice. Gentle shaking can be performed during the trypsin digest to help facilitate separation of the blood vessels from the remaining tissue, but is not necessary. It is important to avoid strong agitation as this can lead to tearing of the vessels.

The critical steps that require the most attention involve separating the vasculature after trypsin digestion. When performing the water washes, it is important to avoid pipetting the vasculature. Pipetting of the vasculature should only be used as a method of separating the vasculature after the walls have been coated with trypsin. All manipulations need to be done carefully under the dissection microscope as the network of vessels can be easily damaged. The different techniques described in step 4 can be used successfully in a variety of combinations. Ultimately, it will be up to the individual experimenter to find the approach that works best for them. It is important that all tools that will come into contact with the blood vessels be coated with trypsin. Dipping the tools intermittently and often into trypsin during the procedure can prevent adhesion of vasculature and potential disruption.

The efficacy of the procedure is also dependent on the background mouse model. Some mouse backgrounds are not as conducive to preserving the whole vasculature network. For example, the FVB background is homozygous for a phosphodiesterase-6 mutation making them prone to retinal degeneration [17]. From our experience, we have found that their retinal vasculature is inherently more fragile, and we were unable to preserve the entire vasculature network. Thus, it is important that the background be taken into consideration prior to attempting this technique.

Ultimately, the main limitation to this procedure is an individual's dissection skills. It is important to note that even if an individual knows the techniques well, it may take several attempts before they begin to achieve consistent results. Thus, we would recommend that the interested lab personnel perform several practice dissections to gain confidence and arrive at the best and most consistent approach prior to performing the procedure on their experimental tissue. It is important to note that, once mastered, trypsin digest is a valuable technique that can be used to assess vascular pathology (**Figure 4**). Trypsin digest can also be performed successfully on preserved retinas that have been in fixative for several years [1].

In conclusion, trypsin digest continues to be an extremely useful method to analyze the retinal vasculature since its description by Cogan and Kuwabara in the 1960s. It allows detailed visualization of the entire vascular network, as compared to immunofluorescence/H&E/PAS stained sections, flat-mounts and dye-injections. Although it is technically difficult, this manuscript describes the procedure in detail so that it can be performed successfully. As a result, we hope investigators novice to the method will have sufficient guidance to allow successful and consistent analysis of the retinal vasculature in their experimental models.

Disclosures

The authors declare that they have no competing financial interests.

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