Published in final edited form as:

Methods Mol Biol. 2012 ; 849: . doi:10.1007/978-1-61779-551-0_28.

Histological Staining of Amyloid and Pre-Amyloid Peptides and Proteins in Mouse Tissue

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Abstract

The increased availability of transgenic mouse models for studying human diseases has shifted the focus of many laboratories from in vitro to in vivo assays. Herein, methods are described to allow investigators to obtain well preserved mouse tissue to be stained with the standard histological dyes for amyloid, Congo Red and Thioflavin S. These sections can as well be used for immunohistological procedures that allow detection of tissue amyloid and pre-amyloid, such as those composed of the amyloid- β peptide, the tau protein, and the islet amyloid polypeptide.

Keywords

Mouse; perfusion; brain; pancreas; fixation; histology; Congo Red; Thioflavin S; immunohistochemistry; amyloid- β ; plaques; tau; tangles; islet amyloid polypeptide

1. Introduction

Over the last couple of decades, numerous transgenic mouse models have been developed for Alzheimer's disease and other amyloid-related disorders. The increased availability of these models continues to shift the focus of many laboratories from in vitro and cell-based assays to animal models. Several tissue preparation methods, histological stains and immunohistochemical procedures can be used for amyloid and pre-amyloid detection, and have been described in varying details by others (1–10). Here, a combined protocol is provided (11–14), that focuses on these methods applied to fixed mouse tissue sections (brain and pancreas). First of all, appropriate tissue preservation is fundamental for any following steps; systematic sectioning and storage of tissue sections is as well important for subsequent analysis. Congo Red and Thioflavin S are the two major histological stains used to detect any form of amyloid. These dyes bind to the characteristic β -pleated sheet conformation of amyloid. Additionally, monoclonal mouse antibodies are increasingly employed to detect pre-amyloid and amyloid deposits and their use has led to the development of kits that reduce non-specific binding of these antibodies to mouse tissue. Although these kits come with detailed protocols, the exact procedure often has to be adjusted depending on the tissue preparation, the antigen and the monoclonal antibodies. Interestingly, less costly reagents can provide similar or better results, at least for certain antibodies.

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³³Be sure to use DAB in its salt form (tetrahydrochloride) or it will not dissolve.

2. Materials

1. Sodium pentobarbital.
2. Surgical instruments: Scissors, various forceps, feeding needles for newborn mice or birds.
3. Perfusion pump with variable flow (Mini-Pump, Variable flow, Fisher Scientific, Morris Plains, NJ, #138761), with tubing (inner diameter: 0.38 mm).
4. 0.1 M sodium/potassium phosphate buffer, pH 7.4: Dissolve 4.03 g KH_2PO_4 monobasic and 10 g Na_2HPO_4 dibasic, anhydrous in distilled water, pH to 7.4 with dilute NaOH or HCl, fill to 1 liter with distilled water.
5. Heparin (1000 USP units/ml).
6. 4% paraformaldehyde in 0.1 M sodium/potassium phosphate buffer: Prepare 0.2 M sodium/potassium phosphate buffer (8.06 g of KH_2PO_4 monobasic and 20 g of Na_2HPO_4 dibasic, anhydrous dissolved in distilled water, pH to 7.4 and adjust volume to 1 liter). A portion of this solution can be diluted to 0.1 M for use in mouse perfusions. The following steps should be performed in a chemical hood. a) Weigh 40 g of prilled paraformaldehyde. b) Heat 450 ml of distilled water to 55°C, remove from heat and add paraformaldehyde while stirring. c) Add 10 N NaOH until the solution clears which occurs when the fixative dissolves (see Note 1). d) Allow to cool to room temperature and then filter through Whatman #1 (see Note 2). Add water to 500 ml and then add equal volume of 0.2 M phosphate buffer to obtain 1 liter of the fixative. Use freshly prepared and/or store refrigerated (see Note 3).
7. Conical tubes, 15 ml.
8. 20% glycerol/2% dimethylsulfoxide in 0.1 M sodium/potassium phosphate buffer, pH 7.4: Prepare buffer as listed above, then add 200 ml of glycerol and 20 ml of dimethylsulfoxide to a total volume of 1 liter.
9. Single edge razor blades No. 9.
10. Paraffin embedding facility (for pancreatic tissue)
11. Water bath with a temperature controller for mounting paraffin embedded sections
12. Tissue Freezing Medium® for frozen tissue specimens.
13. Paint brushes, size 0 and 1.
14. 24 well tissue culture plates.
15. Ethylene glycol based cryoprotectant: 30% sucrose, 30% ethylene glycol in 0.1 M sodium/potassium phosphate buffer, pH 7.4. Prepare the buffer as listed above, then add 300 g of sucrose and 300 ml ethylene glycol to a total volume of 1 liter.
16. Microscope slides (25 × 75 × 1.0 mm).
17. 95% ethanol : 1.0 M acetic acid (1:1).

¹Stop adding NaOH when it does not enhance the clarity of the solution. Powdered paraformaldehyde is more soluble than the prilled version but is not recommended because of enhanced risk of exposure to airborne particles. Although the prilled fixative may not dissolve completely, undissolved material can be filtered away.

²It is recommended to use double filter paper or to allow the solution to cool down. Warm solution may rupture the filter paper.

³It is usually recommended to use freshly prepared fixative but we have not noticed any differences in the quality of tissue sections or amyloid staining when the fixative has been refrigerated for up to several weeks prior to use.

18. Gelatin.
19. Chromium potassium sulfate.
20. 1% Congo Red solution in 50% ethanol: Stir 2.5 g of Congo Red into 250 ml of 50% ethanol.
21. 100% ethanol.
22. Xylene or CitriSolv® (Fisher Scientific).
23. Saturated lithium carbonate solution: Stir 2.6 g of lithium carbonate into 200 ml of distilled water.
24. Cell strainers 100 μ m nylon, (Falcon, No. 352360, BD Biosciences, Bedford, MA) or equivalent.
25. 6 well culture dishes.
26. MOM® kit (Vector Laboratories, Burlingame, CA) or equivalent.
27. Citrate buffer (10 mM Citric Acid, 0.05 % Tween 20, pH 6.0): Dissolve 1.92 g of anhydrous citric acid and 0.5 ml of Tween 20 in 1000 ml of distilled water.
28. 80% formic acid
29. Vectastain ABC Elite kit to detect rabbit IgG primary antibodies (Vector) or equivalent.
30. Primary antibody diluent in phosphate buffered saline for polyclonal antibodies: 2% Triton X-100, 0.1% sodium azide, 0.01% bacitracin, 2% bovine serum albumin, and 10% normal serum from the same species that the secondary antibody is raised in.
31. Phosphate buffered saline: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride [PBS tablets (Sigma, St. Louis, MO): dissolve 1 tablet in 200 ml of water].
32. 0.3% hydrogen peroxide in phosphate buffered saline (for quenching) or in 0.2 M sodium acetate buffer (for reacting with diaminobenzidine tetrahydrochloride), made fresh from 30% solution.
33. Various antibodies against amyloid- β (A β), tau and islet amyloid polypeptide (IAPP).
34. 0.2 M sodium acetate buffer, pH 6.0, adjust pH with 1 M acetic acid.
35. Diaminobenzidine tetrahydrochloride.
36. Nickel ammonium sulfate.
37. Filter disks (0.45 μ m).

3. Methods

The methods described below outline (1) a mouse perfusion technique and tissue sectioning (brain and pancreas), (2) preparation of gelatin-coated slides, (3) two amyloid histology procedures, and (4) an immunohistological method for staining of pre-amyloid and amyloid deposits in mouse tissue (A β , tau and IAPP).

3.1. Mouse Perfusion and Tissue Sectioning

1. The mouse has to be deeply anaesthetized by intraperitoneal injection of sodium pentobarbital (150 mg/kg).
2. The mouse is subsequently placed over a collection container on a tray or grid that will allow the perfusate to drain into the container (see Note 4).
3. The chest cavity is then exposed and the left ventricle is punctured with pointed forceps.
4. A perfusion cannula is then placed into the ventricle and held in place with hemostats or with forceps that can be secured with a paperclip (see Note 5). The right atrium is then cut and the perfusion pump started. Cutting the atrium allows the perfusate to exit the circulatory system (see Note 6).
5. The initial perfusion should be performed with a physiological solution such as 0.1 M sodium/potassium phosphate buffer or phosphate buffered saline, pH 7.4.
6. Heparin (1 unit/g bodyweight) may be added to the perfusion solution to eliminate blood clotting (see Note 7). This is especially helpful for investigators that are still learning the perfusion technique.
7. If biochemical analysis is needed on the tissue, the brain can be removed from the skull following perfusion with 25–50 ml of phosphate buffer (5–10 minutes or until the perfusate runs clear). The whole brain or its portion can then be snap frozen on dry ice (see Note 8). The remaining portion can then be immersion fixed in 4% paraformaldehyde overnight. If the whole brain is to be used for histology, the perfusion has to be temporarily halted while the tubing is transferred to a 4% paraformaldehyde solution. The same applies to the pancreas (see Note 9).
8. The perfusion is then resumed with about 50–100 ml of fixative. The fixative will shrink the brain which will aid in removing it from the skull, although with experience an unfixed brain can easily be removed intact.
9. The brain/pancreas can then be post-fixed overnight at 4°C in the same fixative (see Note 10).
10. The brain is subsequently transferred to 20% glycerol/2% dimethylsulfoxide in 0.1 M phosphate buffer and kept refrigerated overnight. (see Note 11).

⁴It is convenient to perform this procedure over a sink but the perfusate should be disposed of according to institutional guidelines.

⁵As a perfusion cannula, 22 gauge feeding needle for newborn mice or birds is recommended. Blunted needles as these are less likely to tear the heart during perfusion. The cannula will fit onto a regular intravenous tubing extension set that also has an injection site for the heparin solution. Alternatively, heparin can be added directly to the perfusion solution.

⁶The lungs will expand if there is a buildup of fluids within the circulatory system. The cause is usually that efflux of the perfusate out of the right atrium is blocked and/or the cannula has accidentally been placed in the right ventricle so the perfusion liquid will enter the lungs first instead of the main circulation.

⁷Blood clots will give non-specific staining in immunohistochemistry.

⁸If one hemisphere needs to be saved for biochemical analysis it is convenient to place the brain on a cold surface and cut the brain slightly off the midline with a single-edged razor blade. The larger portion should be immersion fixed and it will contain a fraction of the contralateral hemisphere. However, if the brain is cut through the midline between the hemispheres, the hippocampus will often separate from the brain section during staining and/or mounting onto the slides.

⁹It can be difficult to distinguish the pancreas from surrounding fat tissue, particularly in older mice that often develop substantial abdominal adipose tissue. Obesity is also induced to promote pancreatic deposition of IAPP aggregates in mouse models which makes this more of an issue. The pancreas is located at the hilus of the spleen and it is best to remove those together. Close examination will reveal that the texture and consistency of the pancreatic tissue is different from associated adipose tissue and these are more easily distinguished after fixation and very clearly with microscopical evaluation of histological sections.

¹⁰If the tissue is fixed by perfusion, post-fixation for 6–8 hours may be sufficient but overnight fixation does not affect amyloid or pre-amyloid staining.

¹¹Without this step, the brain may crack while being frozen for sectioning.

11. The pancreas needs to be embedded in paraffin before sectioning. Hence, when fixed it cannot go into a sugar solution (such as glycerol- or sucrose solution) as it interferes with the paraffin embedding and subsequent sectioning. Instead, place the fixed pancreas in PBS and deliver to the in-house paraffin embedding facility (see Note 12)
12. The brain can then be sectioned at 40 μm on a freezing microtome or in a cryostat (see Note 13). The paraffin embedded pancreas is ideally sectioned at 5–10 μm and mounted directly onto slides in a heated water bath (37–41°C; see Note 14).
13. It is often necessary to save all the sections and this can be done conveniently by placing them into series in 24 well cell culture tray filled with ethylene glycol cryoprotectant. We usually save sections from each brain into five series at 200 μm intervals. The sections are then stored at –20°C until used (see Note 15).
14. Sections from the pancreas are taken as needed from the paraffin block which is stored at room temperature.

3.2. Preparation of gelatin coated slides

For immunohistochemical procedures, commercially available coated slides are sufficient but for Congo Red staining of slide-mounted sections, it is preferable to use gelatin-coated slides.

1. Use commercially available pre-cleaned slides (75 × 25 mm).
2. Place in a slide rack (see Note 16)
3. Rinse in distilled water.
4. Dip for one minute in 1:1 solution of 95% ethanol and 1.0 M acetic acid.
5. Rinse in distilled water until water sheets of slides (15 to 30 seconds).
6. Dip for two minutes in freshly prepared filtered gelatin solution. Prepare by heating 300 ml of distilled water to 60°C, then add 3 g of gelatin and stir. When fully dissolved, add 0.3 g of chromium potassium sulfate to harden the gelatin and stir until dissolved. Filter through Whatman #1 or equivalent filter paper before use.
7. Dry at 30–50°C overnight.
8. Place in slide containers until used.

3.3. Congo Red staining

1. Prepare a saturated Congo Red solution by adding 2.5 g of Congo Red to a total volume of 250 ml of 50% ethyl alcohol. Undissolved material should be filtered through a Whatman #1 or equivalent filter paper (see Note 17). It is preferable to perform this staining on sections that have been mounted onto slides (see Note 18).

¹²Nowadays, only a few laboratories have paraffin embedding stations but most institutions have central facilities for this procedure, which is much more cost effective and should provide more consistent results.

¹³By sectioning the brain at 40 μm , the sections will withstand free-floating staining procedures. Paint brush #1 is convenient to use to move the sections from the blade into the ethylene glycol cryoprotectant solution. Tissue Freezing Medium® or equivalent is used to secure the brain to the specimen holder. It usually takes less time to section the brain on a freezing microtome than in a cryostat.

¹⁴If the paraffin sectioned tissue does not spread well on the slide when mounted, the temperature of the water bath used for mounting the tissue should be increased gradually from 37°C until ideal conditions are achieved. This typically occurs prior to 41°C.

¹⁵Prolonged storage at –20°C to –30°C does not seem to affect staining of amyloid or pre-amyloid.

¹⁶The slide rack should have a handle and an open bottom so that the rack can be moved quickly between solutions (equivalent to Wheaton No. 900200 that includes a staining dish that fits the rack). Nineteen slides will fit in each rack if placed in a zig-zag way. Larger racks can also be used.

2. After defatting the tissue in xylene or CitriSolv®, it is hydrated by taking it through a series of ethyl alcohol solutions (100%, 95%, 80% and 70%, 1–2 minutes in each) before staining in the Congo Red solution for 1 hour.
3. The slides are subsequently dipped into a saturated lithium carbonate solution for 10–20 seconds and then rinsed for a similar period in tap water or distilled water (see Note 19).
4. Dip the slides for 5–60 seconds in 70% ethanol to get rid of some of the background staining, and then transfer the slides quickly through 80%, 95%, and two sets of 100% ethanol (15 seconds to 2 minutes in each) before placing them in xylene or CitriSolv for 5–10 minutes (see Note 20).
5. The slides can now be coverslipped with mounting media such as DePex (see Note 21).
6. Congo Red staining can then be viewed under plane-polarized light. Amyloid plaques should give apple-green birefringence, usually as a Maltese cross, whereas the neurons that are non-specifically stained will not emit birefringence (see Note 22, and Fig. 1). For an alternative Congo Red staining procedure, please refer to Chapter 24.

3.4. Thioflavin S staining

1. As with the Congo Red staining method, it is preferable to stain mounted sections with Thioflavin S.
2. Prepare 1% Thioflavin S solution in distilled water, and filter it through Whatman #1 before use (see Note 23).
3. Sections are defatted in xylene or CitriSolv (5–10 minutes) and then hydrated through a series of ethyl alcohol solutions (100%, 95%, 80%, 70%, 1–2 minutes in each one), placed for a few seconds in water and then stained with 1% Thioflavin S for 30–60 minutes (see Note 24).

¹⁷If the staining will be performed in regular glass slide container, a total volume of 200–250 ml will be sufficient to cover the slides. The solution should be stirred for a few hours or even overnight to ensure that most of the chemical is dissolved. Preferably use freshly made solution. After sitting at room temperature for several days or weeks, the stain will not work properly.

¹⁸Commercially available coated slides, such as Fisherbrand® Superfrost®/Plus work fine for immunohistochemistry but for histological stains such as Congo Red or Cresyl Violet, it is preferable to use gelatin coated slides for improved adherence of the sections to the slide.

¹⁹Some protocols suggest washing the sections in running tap water for 15 minutes. This may be appropriate for human sections but in our experience, mouse and rat sections partially come off the slides when left in water for too long.

²⁰Protocols for Congo Red staining usually involve counterstains such as Harris hematoxylin but this procedure can be avoided by not washing off the non-specific Congo Red neuronal staining. Most of this staining comes off in the 70% ethanol so care should be taken to keep the slides only briefly in this solution. The exact time varies a bit but 5 to 60 seconds is usually sufficient to reduce the background staining. The remaining intensity should be similar to Cresyl Violet staining. The sections can always be restained in the Congo Red solution if they become too destained in the alcohol solutions. Leave the slides for at least two minutes in the final set of 100% ethanol. Water will turn the xylene or Citrosolv® cloudy and can interfere with microscopic analysis of the tissue sections.

²¹Various mounting media can be used. We are particularly fond of DePex mounting media. It does not shrink much while setting which reduces bubble formation and it does not come off in sharp flakes when the slides are cleaned with a razor blade after it has set overnight. It is best to use coverslips (22 × 60 mm) that are slightly smaller than the slide (25 × 75 mm). The slides will be easier to clean and bubbles are less likely to form.

²²Birefringence is often seen in larger blood vessels and in threads associated with the surface of the brain. This observation does not confirm the presence of amyloid and can be due to Congo Red binding to collagen which is known to result in birefringence.

²³As with Congo Red, a volume of 200–250 ml is sufficient if standard glass staining containers are used. Similar results can be obtained with 0.5% Thioflavin S solution.

²⁴Less staining time at lower dye concentrations may provide equivalent results, depending on the tissue and the thickness of the sections. For example, for 10 µm thick paraffin embedded sections of the mouse pancreas, incubation for 2 minutes in 0.5% Thioflavin S solution is sufficient for staining IAPP deposits.

4. The sections are subsequently dehydrated through a series of ethyl alcohol solutions (70%, 80%, 95%, 100%, 100%, 1–2 minutes in each one) and then placed in xylene or CitriSolv® (5–10 minutes) before being coverslipped with DePex mounting media.
5. The slides are then cleaned as described above for the Congo Red method before being viewed under fluorescence using the filter set recommended for that particular microscope. Thioflavin S bound to amyloid will emit fluorescence (see Note 25 and Fig. 1).
6. The stained sections should be stored in a cool, dark place such as a refrigerator.

3.5. Immunohistochemistry – Staining for amyloid and pre-amyloid

The following protocols are for free floating mouse brain sections (see Note 26). Initially, certain controls should be included such as pre-adsorption of the primary antibody with the antigen and omitting of the primary and secondary antibodies as well as the avidin-peroxidase complex. For mouse-on-mouse detection it is appropriate to include omission of the primary antibody in each run. For optimal immunodetection of A β plaques it is not necessary to pretreat the sections with heat or formic acid as is needed for human sections. Likewise, for tau staining, pretreatment is not necessary in mouse sections. However, for IAPP staining of the mouse pancreas, epitope unmasking by boiling in citrate buffer is recommended as detailed below.

3.5.1. Mouse monoclonal antibodies—To eliminate background staining it is recommended to use MOM® kit (Vector Laboratories) or equivalent reagents. We have modified the kit instructions to our conditions. It is likely that further adjustments can be performed to save reagents. At least for certain tau antibodies, less expensive reagents work as well or better (see Note 27).

A β staining

1. Sections are removed from the ethylene glycol cryoprotectant and washed three times for 10 minutes each in PBS.
2. The sections are treated for 30 minutes in 0.3% hydrogen peroxide, freshly prepared from a 30% solution, to quench endogenous peroxidase activity.
3. Following two 5 minute washes in PBS, the sections are placed in MOM blocking reagent (Vector Laboratories) for 1 hour (5 drops per 10 ml of PBS; see Note 28), and then washed twice for 5 minutes in PBS.
4. The sections are then placed for 1 hour or longer in mouse primary antibody in MOM diluent (see Note 29). The diluent is prepared by adding 1200 μ l of protein concentrate from the MOM kit into 15 ml of PBS. Control sections are placed in MOM diluent alone.

²⁵FITC filter which commonly exists in many microscopes works well although the Thioflavin wavelength is slightly different.

²⁶A similar protocol can be used for slide mounted sections although incubation periods may have to be increased to allow sufficient penetration of the antibodies. It is most convenient to use cell strainers, with 100 μ m nylon mesh, to move the sections between solutions. The strainers can be placed in 6 well culture dishes for all the washes. To save material, for all other incubations, the strainers can be placed in small individual Petri dishes of a similar diameter as the wells. Less volume is needed in these dishes to immerse the sections.

²⁷For example, PHF1 antibody staining of normal mouse tissue can be reduced/eliminated by using the procedure described by Peter Davies (15). For the MC1 antibody, we obtained comparable results with the two methods (Vector MOM kit vs. Davies' protocol), with the latter using less expensive reagents.

²⁸The MOM blocking solution can be used at least few times.

²⁹The proper dilution of the monoclonal antibodies will have to be assessed.

5. After two 5 minute washes in PBS, the sections are incubated for 1 h in biotinylated anti-mouse IgG (secondary antibody) solution that is prepared by adding 20 μ l of stock solution into 15 ml of MOM diluent (see Note 30).
6. Following two 5 minutes washes in PBS, the sections are placed for 30 minutes in avidin-peroxidase solution that is prepared 30 minutes in advance by adding 2 drops of Reagent A and 2 drops of Reagent B into 40 ml of PBS.
7. Subsequently, the sections are washed for 5–10 minutes in 0.2 M sodium acetate solution, pH 6.0 (see Note 31), and then reacted with the chromogen of choice. We usually use diaminobenzidine tetrahydrochloride (DAB) as a chromogen and nickel ammonium sulfate to intensify the reaction. This solution can be prepared by dissolving 35 mg of DAB in 100 ml of 0.2 M sodium acetate buffer, pH 6.0. Nickel ammonium sulfate (2.5 g) is subsequently added and once dissolved the solution is filtered through 0.45 μ m filter disk by using a 20–60 ml disposable syringe (see ^{32–34}).
8. When in the DAB solution, 20 – 40 μ l of 0.3% hydrogen peroxide (see Note 35) is gradually added to each reaction well (5–10 ml) and the staining reaction allowed to proceed until the sections have reached the desired staining intensity (5–10 minutes; see Note 36). The sections are then washed in 0.2 M sodium acetate buffer and subsequently transferred to PBS for mounting onto slides (see Note 37).

Tau staining: For many tau antibodies, the same procedure as described for A β can be used except that TBS is recommended in place of PBS for tau phospho-specific antibodies. However, as indicated above, the use of less expensive reagents may give better or equivalent results (see Note 27).

IAPP staining

1. Deparaffinize sections in two changes of xylene, 5 minutes each.
2. Hydrate in two changes of 100% ethanol for 3 minutes each, then in 95% and 80% ethanol for 1 minute each. Then rinse in distilled water.
3. Immerse the slides in a slide holder for 10 minutes in a beaker containing boiling sodium citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) (see Note 38).
4. Let the sections adjust for 20 minutes at room temperature.
5. Treat the tissue for 10 minutes with 80% formic acid (see Note 39).
6. Wash sections twice in PBS with 0.1% Tween 20.

³⁰We have also diluted the secondary antibody into PBS with similar results.

³¹It is convenient to prepare 1.0 M sodium acetate, pH 6.0 stock solution. pH can be adjusted with 1.0 M acetic acid. This solution is best kept refrigerated. The 0.2 M solution can be prepared from the stock in 500 ml aliquots and stored refrigerated.

³²The syringes can be used repeatedly and discarded when it becomes difficult to push the plunger through the syringe, which will occur over time.

³⁴DAB should not be dissolved in PBS or any other phosphate buffer as phosphates will precipitate it out of solution and these aggregates can then adhere to the sections leading to non-specific staining. Likewise, when staining is done, the sections should not be moved directly into PBS but rather washed first in 0.2 M sodium acetate to remove any residual DAB.

³⁵Likewise, hydrogen peroxide used for the reaction should be diluted in the 0.2 M sodium acetate buffer and not in PBS. We usually dilute 10 μ l of 30% hydrogen peroxide into 1 ml of the buffer, and then add 20–40 μ l of this solution (0.3% hydrogen peroxide) to the reaction (5–10 ml).

³⁶If the sections reach the desired staining intensity very quickly (seconds to a few minutes), further dilution of the primary and/or secondary antibodies is recommended to preserve these reagents. Typically, 7–8 minutes staining period is ideal.

³⁷The sections can be stored overnight or over a weekend in the PBS solution at 4–6°C prior to mounting. Longer storage is not recommended as increased hydration of the sections makes mounting onto slides more difficult.

³⁸Some unmasking protocols suggest boiling the sections for 20–40 minutes but this is not necessary to unmask IAPP epitopes in the mouse pancreas. Such a long boiling period causes the mouse tissue to come off the slides and should be avoided.

7. Then proceed in the same manner as described in the A β staining protocol, starting with treating the sections in 0.3% hydrogen peroxide.

Polyclonal antibodies: When rabbit polyclonal antibodies are used on mouse brain sections, background staining due to cross-reactivity is less of an issue than with mouse antibodies. Regular secondary antibody kits can be used such as the Vector Elite kit for rabbit IgG antibodies. For A β and IAPP, this protocol is the same as the mouse monoclonal protocol except that the primary antibody diluent is different (as detailed in Materials) and the secondary antibody is diluted into PBS (see Note 40).

Acknowledgments

This work was supported by NIHs grant AG20197, AG032611 and DK075494 and the Alzheimer's Association. These protocols were in part adapted from methods obtained from Stanley A. Lorens and Debra Magnuson at Loyola University Chicago. We thank Drs. Fernando Goni and Ayodeji Asuni for their comments on the original manuscript, which has now been substantially updated and expanded in this 2nd edition of the book.

References

1. Kitamoto T, Ogomori K, Tateishi J, Prusiner SB. Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. *Lab Invest.* 1987; 57:230–236. [PubMed: 2441141]
2. Davies L, Wolska B, Hilbich C, Multhaup G, Martins R, Simms G, Beyreuther K, Masters CL. A4 amyloid protein deposition and the diagnosis of Alzheimer's disease: prevalence in aged brains determined by immunocytochemistry compared with conventional neuropathologic techniques. *Neurology.* 1988; 38:1688–1693. [PubMed: 3054625]
3. Gentleman SM, Bruton C, Allsop D, Lewis SJ, Polak JM, Roberts GW. A demonstration of the advantages of immunostaining in the quantification of amyloid plaque deposits. *Histochemistry.* 1989; 92:355–358. [PubMed: 2478507]
4. Lamy C, Duyckaerts C, Delaere P, Payan C, Fermanian J, Poulain V, Hauw JJ. Comparison of seven staining methods for senile plaques and neurofibrillary tangles in a prospective series of 15 elderly patients. *Neuropathol Appl Neurobiol.* 1989; 15:563–578. [PubMed: 2482455]
5. Wisniewski HM, Wen GY, Kim KS. Comparison of four staining methods on the detection of neuritic plaques. *Acta Neuropathol (Berl).* 1989; 78:22–27. [PubMed: 2472039]
6. Vallet PG, Guntern R, Hof PR, Golaz J, Delacourte A, Robakis NK, Bouras C. A comparative study of histological and immunohistochemical methods for neurofibrillary tangles and senile plaques in Alzheimer's disease. *Acta Neuropathol (Berl).* 1992; 83:170–178. [PubMed: 1373017]
7. Raskin LS, Applegate MD, Price DL, Troncoso JC, Hedreen JC. Comparison of new and traditional methods for detection of senile plaques in Alzheimer's disease. *J Geriatr Psychiatry Neurol.* 1995; 8:125–131. [PubMed: 7794478]
8. Cullen KM, Halliday GM, Cartwright H, Kril JJ. Improved selectivity and sensitivity in the visualization of neurofibrillary tangles, plaques and neuropil threads. *Neurodegeneration.* 1996; 5:177–187. [PubMed: 8819139]
9. Shiurba RA, Spooner ET, Ishiguro K, Takahashi M, Yoshida R, Wheelock TR, Imahori K, Cataldo AM, Nixon RA. Immunocytochemistry of formalin-fixed human brain tissues: microwave irradiation of free-floating sections. *Brain Res Brain Res Protoc.* 1998; 2:109–119. [PubMed: 9473616]

³⁹We have noticed that IAPP staining is improved when the boiling period is followed by 10 minutes formic acid treatment. Longer treatment should be avoided as the tissue can then come off the slides. This improvement may be antibody dependent and the necessity of this additional step should be evaluated for each antibody.

⁴⁰For general immunohistochemistry we have often used 0.3% Triton-X in PBS as the wash solution and to dilute the secondary antibody as well as the A+B solution. The detergent may enhance epitope exposure which facilitates detection of certain antigens but is not necessary for staining amyloid plaques in mouse section. The primary antibody diluent can be stored refrigerated prior to use for at least several months.

10. Cummings BJ, Mason AJ, Kim RC, Sheu PC, Anderson AJ. Optimization of techniques for the maximal detection and quantification of Alzheimer's-related neuropathology with digital imaging. *Neurobiol Aging*. 2002; 23:161–170. [PubMed: 11804699]
11. Sigurdsson EM, Lorens SA, Hejna MJ, Dong XW, Lee JM. Local and distant histopathological effects of unilateral amyloid- β 25–35 injections into the amygdala of young F344 rats. *Neurobiol Aging*. 1996; 17:893–901. [PubMed: 9363801]
12. Sigurdsson EM, Lee JM, Dong XW, Hejna MJ, Lorens SA. Bilateral injections of amyloid- β 25–35 into the amygdala of young Fischer rats: Behavioral, neurochemical, and time dependent histopathological effects. *Neurobiol Aging*. 1997; 18:591–608. [PubMed: 9461057]
13. Sigurdsson EM, Scholtzova H, Mehta PD, Frangione B, Wisniewski T. Immunization with a non-toxic/non-fibrillar amyloid- β homologous peptide reduces Alzheimer's disease associated pathology in transgenic mice. *Am J Pathol*. 2001; 159:439–447. [PubMed: 11485902]
14. Asuni AA, Boutajangout A, Quartermain D, Sigurdsson EM. Immunotherapy targeting pathological tau conformers in a tangle mouse model reduces brain pathology with associated functional improvements. *J Neurosci*. 2007; 27:9115–9129. [PubMed: 17715348]
15. Davies, P. Tau immunohistochemistry protocol: Mice. <http://alzforum.org/res/com/protocol/protocol.aspx?pid=3> Accessed 25 March 2011

Figure 1.
Coronal brain sections depicting cortical regions (50X) in a 12 month old 5XFAD mouse stained with 6E10/4G8 (A), Thioflavin S (B) and Congo Red (C). D, E and F are magnifications (400X) of the boxed areas in A, B, and C, respectively. Numerous A β plaques are visible, many of which are Thioflavin S and Congo Red positive.