Video Article

Isolation, Fixation, and Immunofluorescence Imaging of Mouse Adrenal Glands

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Abstract

Immunofluorescence is a well-established technique for detection of antigens in tissues with the employment of fluorochrome-conjugated antibodies and has a broad spectrum of applications. Detection of antigens allows for characterization and identification of multiple cell types. Located above the kidneys and encapsulated by a layer of mesenchymal cells, the adrenal gland is an endocrine organ composed by two different tissues with different embryological origins, the mesonephric intermediate mesoderm-derived outer cortex and the neural crest-derived inner medulla. The adrenal cortex secretes steroids (*i.e.,* mineralocorticoids, glucocorticoids, sex hormones), whereas the adrenal medulla produces catecholamines (*i.e.,* adrenaline, noradrenaline). While conducting adrenal research, it is important to be able to distinguish unique cells with different functions. Here we provide a protocol developed in our laboratory that describes a series of sequential steps required for obtaining immunofluorescence staining to characterize the cell types of the adrenal gland. We focus first on the dissection of the mouse adrenal glands, the microscopic removal of periadrenal fat followed by the fixation, processing and paraffin embedding of the tissue. We then describe sectioning of the tissue blocks with a rotary microtome. Lastly, we detail a protocol for immunofluorescent staining of adrenal glands that we have developed to minimize both non-specific antibody binding and autofluorescence in order to achieve an optimal signal.

Video Link

The video component of this article can be found at https://www.jove.com/video/58530/

Introduction

Immunohistochemistry is a technique for detecting tissue components with the use of antibodies to specific cellular molecules and subsequent staining techniques to detect the conjugated antibodies[1]. This immunohistochemical procedure requires specific fixation and processing of tissues that are often empirically determined for the specific antigen, tissue and antibody utilized[2]. Fixation is crucial to preserve the "original" state of the tissue and thereby maintaining intact cellular and subcellular structures and expression patterns. Further processing and embedding procedures are required to prepare the tissue for sectioning into thin slices that are used for histologic studies involving immunohistochemistry.

Immunostaining can be performed with either chromogenic or fluorescent detection. Chromogenic detection requires the utilization of an enzyme to convert a soluble substrate into an insoluble colored product. While this enzyme can be conjugated to the antibody recognizing the antigen (primary antibody), it is more often conjugated to the antibody recognizing the primary antibody (*i.e.*, the secondary antibody). This technique is highly sensitive; the colored product resulting from the enzymatic reaction is photostable and requires only a brightfield microscope for imaging. However, chromogenic immunostaining may not be suitable when trying to visualize two proteins that co-localize, since the deposition of one color can mask the deposition of the other one. In the case of co-staining, immunofluorescence has proven to be more advantageous. The advent of immunofluorescence is attributed to Albert Coons and colleagues, who developed a system to identify tissue antigens with antibodies marked with fluorescein and visualize them in the sectioned tissues under ultraviolet light[3]. Fluorescence detection is based on an antibody conjugated with a fluorophore that emits light after excitation. Because there are several fluorophores with emissions at different wavelengths (with no or little overlap), this detection method is ideal for the studies of multiple proteins.

The adrenal gland is a paired organ located above the kidney and characterized by two embryologically distinct components surrounded by a mesenchymal capsule. The outer adrenal cortex, derived from the mesonephric intermediate mesoderm, secretes steroid hormones while the inner medulla, derived from the neural crest, produces catecholamines including adrenaline, noradrenaline, and dopamine. The adrenal cortex is histologically and functionally divided in three concentric zones, with each zone secreting different classes of steroid hormones: the outer zona glomerulosa (zG) produces mineralocorticoids that regulate electrolyte homeostasis and intravascular volume; the middle zona fasciculata (zF),

directly beneath the zG, secretes glucocorticoids that mediate the stress response through the mobilization of energy stores to increase plasma glucose; and the inner zona reticularis (zR), which synthesizes sex steroid precursors (*i.e.,* dehydroepiandrosterone (DHEAS))[4].

Some variation in adrenocortical zonation is present between species: for example, *Mus musculus* lacks the zR. The unique postnatal X-zone of *M. musculus* is a remnant of the fetal cortex characterized by small lipid-poor cells with acidophilic cytoplasms[5]. The X-zone disappears at puberty in male mice and after the first pregnancy in female mice, or gradually degenerates in not-bred females[6,7]. Moreover, the tortuosity and thickness of the zG exhibits marked variation between species as does organization of peripheral stem and progenitor cells in and adjacent to the zG. The rat, unlike other rodents, has a visible undifferentiated zone (zU) between the zG and zF that functions as a stem cell zone and/or a zone of transient amplifying progenitors. Whether the zU is unique to rats or simply a more prominently organized cluster of cells is unknown[8,9].

Cells of the adrenal cortex contain lipid droplets that store cholesterol esters that serve as the precursor of all steroid hormones[10,11]. The term "steroidogenesis" defines the process of production of steroid hormones from cholesterol *via* a series of enzymatic reactions that involve the activity of steroidogenic factor 1 (SF1), whose expression is a marker of steroidogenic potential. In the adrenal gland, Sf1 expression is present only in cells of the cortex[12]. An interesting study found the expression of endogenous biotin in adrenocortical cells with steroidogenic potential[13]. While this can be the cause of a higher background in biotin/streptavidin-based staining methods, due to the detection of endogenous biotin by antibody conjugated with streptavidin, this characteristic could be also employed to distinguish the steroidogenic cells from other populations within the adrenal gland, *i.e.,* endothelial, capsular, and medulla cells.

Innervated by sympathetic preganglionic neurons, the adrenal medulla is characterized by basophilic cells with a granular cytoplasm containing epinephrine and norepinephrine. Medulla cells are named "chromaffin" due to the high content of catecholamines that form a brown pigment after oxidation[14]. Tyrosine hydroxylase (TH) is the enzyme that catalyzes the rate-limiting step in the synthesis of catecholamines and, in the adrenal gland, is expressed only in the medulla[15].

Here we present a protocol for the isolation of mouse adrenal glands, their processing for embedding in paraffin and sectioning, and a method to perform immunofluorescence staining on adrenal sections in order to identify the cellular types constituting the adrenal cortex and medulla. This protocol is a standard in our laboratory for immunostaining with multiple antibodies routinely used in our research.

Protocol

All methods were performed in accordance with institutionally approved protocols under the auspice of the University Committee on Use and Care of Animals at the University of Michigan.

1. Preparation for Surgery

1. On the day prior to surgery, prepare 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS). In case of frozen aliquots, proceed to thaw one and store at 4 °C.
   NOTE: 4% PFA is not stable for more than 48 h.
   CAUTION: PFA is toxic, avoid contact with skin and eyes, and dispose in an appropriate container.
2. On the day of the surgery, prepare the surgical instruments by sterilizing the scissors and forceps with 70% ethanol (EtOH) and let them dry on a paper towel.
3. Place a 24-well plate filled with 1x PBS (1 mL/well is sufficient) in an ice bucket.
   NOTE: Adrenals will be kept in this multi-well culture dish after surgery.

2. Adrenal Dissection

1. Euthanize the mouse following standard protocols approved by the Institutional Animal Care and Use Committee (IACUC). A secondary method of euthanasia to ensure death (for example decapitation) is required.
   1. For euthanasia by isoflurane overdose, place the mouse in a chamber filled with isoflurane vapors until respiration ceases, then remove the mouse from the chamber, lay it on a surface and perform cervical dislocation.
      NOTE: Most euthanasia methods cause distress to the animal and are not suitable for stress studies because they may add the confounding variable of activation of the pituitary-adrenal axis and medullary catecholamine release. In this instance, decapitation is the suggested technique to adopt.
2. Lay the mouse supine, sterilize the incision area on the abdomen and flanks with 70% EtOH.
   NOTE: While shaving the fur will increase sterility, for this particular application it is not necessary.
3. Cut the skin in the middle of the abdomen using scissors, separate the skin from the peritoneum, deskin the mouse by pinching the skin around the cut and pulling it in two opposite directions (caudal and rostral). Then cut the peritoneum until reaching the posterior left and right flanks.
4. Remove the murine adrenal gland cutting around the surrounding adipose tissue and place it in the 24-multiwell plate filled with 1x PBS and kept on ice.

3. Peri-adrenal Fat Removal

1. Under a dissecting microscope, place the adrenal on a glass base or a Petri-dish on the stage plate, position the light source, select the magnification and adjust the focus to obtain a clear view of the entire adrenal.
   NOTE: The magnification used can vary depending on personal preferences. A total magnification of 30X with a plan lens 1X, zoom 3X, and eyepiece 10X allows to visualize the whole gland with a good field of view.

2. Quickly remove the adjacent fat tissues with two 25 G needles, avoiding dehydration of the adrenal. If this happens before all fat is removed, move the adrenal back into the well containing 1x PBS for 1–2 min, and then continue with the fat removal process.
3. Wash 2x for 2 min each in 1x PBS.

4. Tissue Processing and Embedding

1. Fix the tissues in 4% PFA/PBS at 4 °C on a rocking platform for 2 h.
2. Remove the 4% PFA and wash the tissues with 1x PBS, 3x for 15 min each at 4 °C.
   CAUTION: PFA is toxic and is a hazardous waste; it should be handled with caution under a chemical hood and disposed into a hazardous waste container.
3. Incubate the adrenals in 50% EtOH at 4 °C on a rocking platform for 2 h.
4. Incubate the adrenals in 70% EtOH at 4 °C on a rocking platform for a minimum of 2 h.
   NOTE: If not proceeding with tissue processing for embedding, adrenals can be stored in 70% EtOH at 4 °C. Avoiding long-term storage in 70% EtOH and proceeding to tissue processing as soon as possible yields better quality samples.
5. Prepare the adrenal for tissue processing by wrapping it in gauze and enclosing it in a tissue embedding cassette. Place the cassette in a jar filled with 70% EtOH and store at 4 °C until ready to move to the tissue processor. Insert the cassette into the tissue processor programmed as reported in **Table 1** and run the program.
6. Transfer the cassette into an embedding station filled with melted paraffin at 65 °C. If a cooling station is not available, position a cold cooling tray next to it.
7. Label an embedding mold and open the tissue embedding cassette. Using a pair of forceps, unwrap the gauze and place the adrenal gently in the desired position at the center of the base in the embedding mold. Pour the melted paraffin on the adrenal. If necessary, hold the adrenal with forceps to secure its position in the mold otherwise it may move around inside the mold.
8. Gently move the embedding mold to the cooling tray and wait until the hardening of the paraffin. After that, to facilitate sectioning, store embedding molds in a cool place.

5. Sectioning with a Rotary Microtome

1. Check that the microtome is clean before starting, brush away all discarded paraffin residues, ensure that the knife (or blade) is sharpened, oil the internal mechanism and fully retract the block holder if necessary.
2. Label a series of microscope slides (positively charged or coated to prevent tissue loss (see **Table of Materials**)), lay them on a slide warmer set at 37 °C, and dispense some autoclaved type 1 ultrapure water on top of each slide.
3. Set section thickness at 5 µm.
4. Insert the microtome blade in the blade holder, control the angle of the blade, make sure that the blade is firmly secured into the holder and check the clearance of the paraffin block and block holder.
5. Bring the block holder to its highest position and, if possible, lock the operating handle, remove the paraffin block from the mold and place it into the block holder securing it firmly with the clamp. The angle of the center line of the blade with the facing surface of the block should be about 20°. If necessary, readjust the paraffin block.
   CAUTION: The blade is sharp.
6. If previously locked, unlock the hand wheel and lower the paraffin block until its face is level with the edge of the microtome blade. Rotate the hand wheel with a steady rhythm. Perform initial trimming to remove the paraffin and to expose the adrenal.
7. Keep turning the hand wheel, and section until the end of the adrenal. Use a brightfield or dissecting microscope to check for the presence of tissue in the sections. Detach the ribbon from the blade and with the help of another pair of forceps place it on the water laid on the glass slide. It may be useful to place the ribbon on a surface, stretch it carefully, and then mount it on the slide.
8. Let the slides dry on the hot plate. Then lay them flat on a slide tray and bake at 37 °C overnight or longer if water is still present. Once dry, store the slides in a slide box at room temperature.
9. Put away all the used equipment and clean the microtome.

6. Immunofluorescence

1. Select the slides for immunostaining and place them in a slide holder.
2. On a hot plate, bring a beaker filled with 0.01M citrate at pH 2.0 to boil. The volume of the buffer depends on the beaker size and must be enough for covering the sections on the slides during boiling (some evaporation needs to be taken into account). Cover the beaker with aluminum foil.
   NOTE: Other methods for antigen retrieval are available (see **Discussion**).
3. Deparaffinize the slides in 100% xylene 2x for 5 min each. Rehydrate the slides using the following series: 100% EtOH 2x for 5 min, 70% EtOH 2x for 5 min, type 1 ultrapure water for 5 min.
   NOTE: After deparaffinization, it is important to not let the sections dry out: tissue sections must always be covered with buffers or solutions until the end of the procedure or the tissue structure can be damaged.
4. For antigen retrieval, place slides in a metal slide holder and insert it in the beaker with boiling citrate without the aluminum foil. Let it boil for 10 min.
5. Remove the beaker from the hot plate and let it cool for 20 min. Make sure that the adrenal sections are still covered with the buffer.
6. Prepare the blocking solution. If using the commercially available staining kit employed for the **Representative Results** (see the **Table of Materials**), in a tube add 1.25 mL of 1x PBS, 1 drop (equal to about 45 µL) of Mouse Ig Blocking Reagent, and 5% of normal goat serum. Store the blocking solution at 4 °C or on ice while working.
   NOTE: The serum used depends on the secondary antibody's host species. Here secondary antibodies raised in goat have been used. For other antibodies, different serum concentrations may be necessary. Empirically determine the correct serum concentration by testing several concentrations.
7. Transfer the slides into a Coplin jar containing 1x PBS and wash 3x 10 min each on a rocker with gentle rocking.

8. Prepare a humidified chamber for the staining.
9. Gently, shake off the PBS from one slide; wipe the slide bottom with a lint-free wipe to remove the excess PBS.
10. Using a special marker for slides with hydrophobic properties, draw a circle surrounding each adrenal section, making sure that the glass surface is dry to avoid spreading of the ink solution to the section. If necessary, carefully wipe dry the surface. Place the slide in the humidified chamber.
11. Quickly, pipette 50 µL (or enough to cover sections) of blocking solution onto every section. Incubate for 1 h at room temperature.
    NOTE: Blocking solution volume needed may vary; it is important that the sections are well covered with the solution.
12. Prepare the diluent solution from the commercially available kit with 7.5 mL of 1x PBS, 600 µL of protein concentrate stock solution and 5% normal goat serum (or any other serum and concentration used in the blocking solution). Store the diluent solution at 4 °C or on ice while working.
13. Prepare the primary antibody solutions diluting the primary antibodies at the appropriate concentrations in the diluent solution. For co-staining, add an aliquot of each antibody in a new tube, and mix gently. Place antibodies and antibody solutions on ice until ready to use.
    NOTE: Here we employed anti-SF1 antibody raised in rabbit and an anti-TH antibody raised in mouse. To prepare the antibody working solutions follow as directed.
    1. For SF1 working solution, in one tube, add 1 µL of anti-SF1 antibody to 999 µL of diluent solution (final concentration of 1.5 µg/mL). For TH working solution, in a tube, add 1 µL anti-TH antibody in 499 µL of diluent solution (estimated final concentration of 2 - 6 µg/mL).
    2. For SF1 + TH antibody working mix, in a fresh tube, pipette 250 µL of SF1 working solution and 250 µL of TH working solution. Mix gently by pipetting. Store on ice.
14. Transfer the slides in a Coplin jar containing 1x PBS and wash 3x for 5 min each on a rocker with gentle rocking.
15. Place the slides back into the humidified chamber after wiping off excess of PBS, pipette the SF1 + TH antibody working mix (or other primary antibody solution) on each section, close the humidified chamber and leave it overnight at 4 °C.
16. On the following day, move the slides into a Coplin jar containing 1x PBS and wash 3x for 15 min on a rocker with gentle rocking.
17. While washing the slides, prepare the secondary antibody solution in the diluent solution using the appropriate concentration. If performing co-staining, add equal amount of each secondary antibody solution in a new tube. Mix gently by pipetting. Store on ice. Protect the tubes from light and store on ice until ready to use.
    NOTE: Here, the secondary antibodies used are 488 nm fluorescent dye-labeled anti-mouse raised in goat and 549 fluorescent dye-labeled anti-rabbit raised in goat. The secondary antibody solution was prepared by adding 0.5 µL of each secondary antibody in 799 µL of diluent solution (final concentrations of 1.2 µg/mL).
18. Place the slides back in the humidified chamber after removing excess of PBS, quickly pipette the secondary antibody solution on the adrenal sections, close the humidified chamber and protect from light. Incubate for 1 h at room temperature.
19. Wash the slides in a Coplin jar with 1x PBS and wash 3x for 15 min on a rocker with gentle rocking, making sure to protect them from light.
20. Prepare the 4',6-diamidino-2-phenylindole (DAPI) solution for nuclear staining: 1 µL of DAPI (20 mg/mL) in 1 mL of 1x PBS.
    NOTE: Blue-fluorescent nuclear counterstain can also be achieved using Hoechst dye.
21. Pipette DAPI solution onto the adrenal sections, cover from light and incubate at room temperature for 7 min.
22. Wash the slides in a Coplin jar with 1x PBS and wash 3x for 5 min on a rocker with gentle rocking while protecting the slides from light.
23. In an area shielded from direct light, lay down a cover glass, and pipette 60 µL or about 3 drops of mounting agent suitable for immunofluorescence along the surface of the cover glass.
24. Gently wipe the back of a slide with a lint-free wipe, position the slide face down toward the cover glass and parallel to it, so the tissue sections face the cover glass with the mounting agent on it. Lightly press the slide on the cover glass and make sure that no air bubble is trapped between the slide and the cover glass.
    1. If necessary, apply further pressure to remove the air bubbles and the excess of mounting agent.
25. Lay down the slide in a dark container and cure at room temperature for a minimum of 24 h (or the time indicated in the information sheet of the mounting agent used) and then proceed to imaging.

7. Imaging

NOTE: A fluorescence microscope connected to a camera is required for detecting and capturing the fluorescence emitted by the tissues after excitation at determined wavelengths. While obvious, it is important to remember to choose the secondary antibody conjugated with fluorochromes whose excitation and emission spectra are compatible with the equipment available. Imaging settings vary according to the microscope and the software used for capturing images. There are some basic rules that apply for imaging adrenal sections, such as making sure that the exposure time, camera gain settings, and light source intensity are kept constant.

1. Turn on the light source and the camera, launch the imaging software following the manufacturer's directions.
   NOTE: The order of these actions can differ depending on the equipment used.
2. Secure the slide on the stage, open the shutter, and looking into the microscope eyepieces use nuclear staining (DAPI) channel and low magnification (4X) to identify the tissue on the slide: adrenal sections are small, and their visualization may require some time.
3. Change to a higher magnification (10X), focus, and close the shutter.
   NOTE: It is important to avoid unnecessarily long exposition under the light that can cause photobleaching, resulting in loss of signal.
4. Using the software commands, select the objective (10X) and the channel in use (DAPI), adjust exposure time (1 s, can be adjusted if the signal is too strong or weak). If available, set binning for live imaging (not acquisition) to '2 X 2', conversion gain to 'mid', readout speed to '20 MHz'. If the software used does not display the scale bar at the end of the imaging, select the scale bar option from the menu and position the bar where desired.
5. Open the shutter, re-adjust the focus if necessary, and switch channels (FITC, TRITC, DAPI). If the microscope is not automated, take a picture of the adrenal in each channel without moving the stage and make sure to adjust the selection of the channel in use. Close the shutter once finished.
   NOTE: Write down the settings employed in case the software does not automatically save them.
6. Save the pictures.

NOTE: Merged pictures can be often created using the microscope's software or other graphics editor software packages.

7. Repeat imaging for other areas of the section and/or with a higher magnification.
   NOTE: A 20X magnification often requires a shorter exposure time (*i.e.*, 800 ms).
8. At the end of imaging, shut off all the equipment in the proper order.

Representative Results

Figure 1 represents a schematic of the entire protocol described above. Adrenal glands are harvested from mice, adjacent adipose tissue is removed under a dissecting microscope, and the adrenal are then fixed in 4% PFA. After this step, adrenals are processed and embedded in paraffin, and sectioned with a microtome to cut the organ into thin slices that are deposited on microscope slides. After drying of the sections, immunofluorescence is carried out and the sections are imaged at the microscope.

The removal of adjacent fat (**Figure 2A**) is important for facilitating further processing and sectioning of the adrenal glands. Successful removal of the surrounding adipose tissue is shown in **Figure 2B**, where the adrenal is easily detectable and no extra fat is visible. During this step it is critical, however, to not let the adrenal gland dry out or this could damage the tissue structure. Dryness is recognizable when the adrenal assumes a wrinkly appearance (**Figure 2C**). This issue can be easily overcome by rehydrating the adrenal in 1x PBS before continuing with the fat removal.

The end results obtained following this protocol are presented in **Figure 3**. The immunofluorescence images illustrate the immunostaining of an adrenal gland at two different magnifications. Red nuclear SF1 staining labels the adrenocortical cells, whereas cytoplasmic green staining labels cells of the medulla. The outer capsule is labeled by nuclear staining in blue (DAPI) since it is not steroidogenic (SF1-negative).

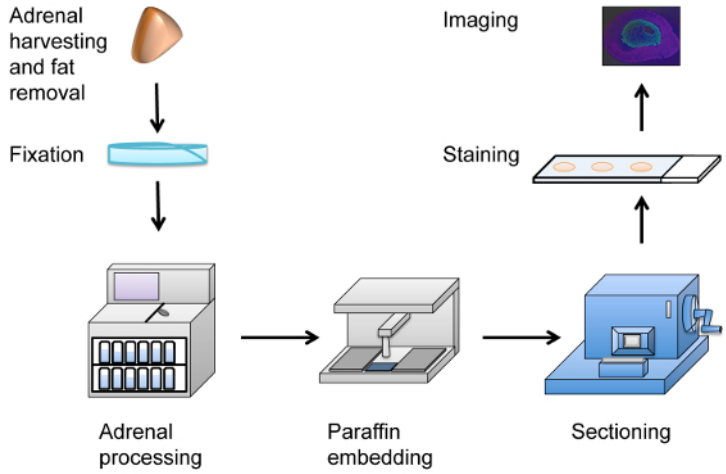

Figure 1: Schematic representation of the protocol. After harvesting the adrenal glands and removing the adjacent adipose tissue, the tissues are fixed in 4% PFA, processed for paraffin embedding, and sectioned. The sections are then immunostained and imaged with a fluorescence microscope. Please click here to view a larger version of this figure.

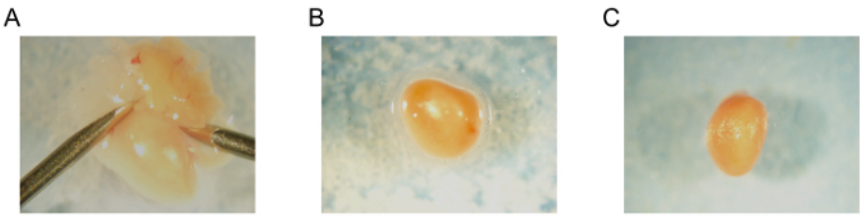

Figure 2: Removal of peri-adrenal fat. (**A**) The fat surrounding the adrenal gland is removed under a dissection microscope. (**B**) Adrenal gland after the clean up. (**C**) Example of tissue drying up and that requires hydration. Please click here to view a larger version of this figure.

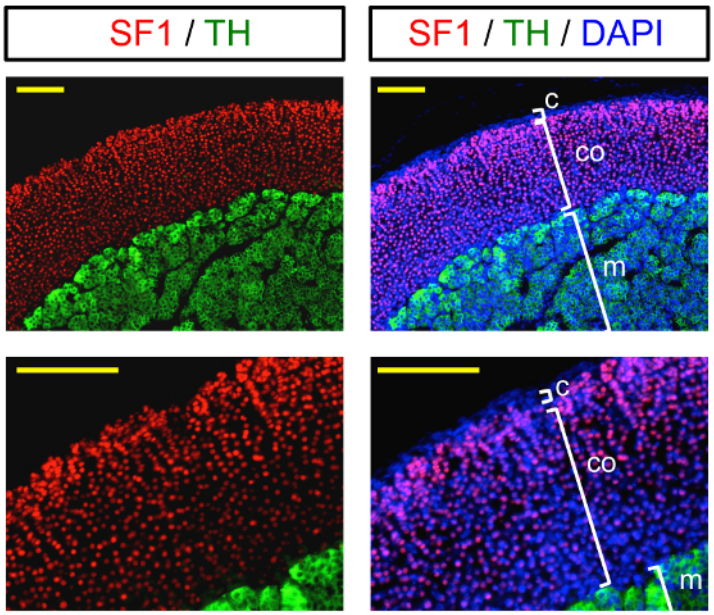

Figure 3: Immunofluorescence imaging of the adrenal gland. Example of immunofluorescence imaging (at different magnifications) of an adrenal gland stained with markers of the adrenal cortex (SF1, nuclear staining) and of the adrenal medulla (TH, cytoplasmic green). Nuclei (DAPI) are labeled in blue. C: capsule; CO: cortex; m: medulla. Scale bars = 200 µm. Please click here to view a larger version of this figure.

Reagent	Station	Temperature	Duration
70% EtOH	1	RT	1 h
90% EtOH	2	RT	1 h
90% EtOH	3	RT	1 h
Absolute EtOH	4	RT	1 h
Absolute EtOH	5	RT	1 h
Absolute EtOH	6	RT	1 h
Absolute EtOH	7	RT	1 h
Xylene	8	RT	1 h
Xylene	9	RT	1 h
Xylene	10	RT	1 h
Paraffin wax	11	62 °C	1 h
Paraffin wax	12	62 °C	1 h
Paraffin wax	13	62 °C	1 h

Table 1: Tissue processor program.

Discussion

This protocol describes a method for the isolation of mouse adrenal glands together with the preparation and staining of sectioned paraffin-embedded mouse adrenals.

Compared to other protocols we tested, this immunofluorescence protocol has proven suitable for the majority of antibodies used in our laboratory. However, in certain cases it may require some adjustments to improve the staining results. One variable that can easily be modified and tested is the length of fixation. In our laboratory, the incubation in 4% PFA can vary from 1 h to 4 h, while in other laboratories the fixation time is extended to 12–24 h[16]. In our hands, however, a longer fixation time led to increased background noise and was not optimal for several antibodies routinely used in our research.

The pH of antigen retrieval can also play a role in the success of staining. Heat-induced epitope retrieval (HIER) can be carried out by employing commercially available solutions with pH ranging from acidic to alkaline, as well as other in-house made buffers such as citrate (pH 6), ethylenediaminetetraacetic acid (EDTA, pH 8), Tris-EDTA (pH 9), Tris (pH 10). Enzymatic treatment can also be an option for antigen unmasking. Incubating the deparaffinized slides for a limited time with a solution containing a proper concentration of an enzyme (for example proteinase K)

can be an alternative method to HIER. It is, however, essential to titrate the enzyme concentration and to determine the ideal incubation time, since over-digestion can negatively impact the tissue[17].

The choice of blocking buffer is also another variable for troubleshooting. Besides the use of normal goat serum and the commercially available blocking buffer mentioned in this protocol (convenient in this instance when using primary mouse antibodies on mouse tissues to prevent high background due to endogenous mouse IgG), there are additional options available such as protein solutions (bovine serum albumin (BSA) or dry milk) or other commercially available buffers. It is critical to make sure that the buffer does not contain substances that can interfere with the staining, such as biotin when using a biotin-streptavidin based method.

The adrenal gland is an endocrine organ characterized by high lipid content that can often make immunostaining challenging due to high autofluorescence of the lipid. Moreover, adrenal cortices from mice and rats are rich in autofluorescent intracytoplasmic lipofuscin, a pigmented granular and amorphous material that ranges in color from yellow to brown. To overcome this problem, compounds such as Sudan Black B (SBB) can quench the fluorescence generated by lipofuscins[18]. Another factor that compromises the outcome of a good staining is the presence of blood cells. Hemoglobin present in the erythrocytes absorbs light of wavelengths <600 nm and can interfere with fluorochromes that span that wavelength[19,20]. While perfusion techniques can be used to clear blood cells from tissue vessels, the use of 10 mM copper sulfate at pH 5 before performing nuclear counterstaining can also help in suppressing the unwanted fluorescence[21].

A critical step in this protocol is the adrenal dissection. The adrenal gland is an organ whose location can be difficult to locate *in situ*: in mice, the glands are small and their position is somewhat variable. Especially in older animals, adrenals are also surrounded by adipose tissue that can interfere with the detection of the glands and their consequent isolation, for this reason it is crucial to have a clear view of the area during this step of the protocol. Removal of peri-adrenal fat removal is a delicate step. Particular attention should be paid to the adrenals while detaching the fat so as not to rupture the capsule. The gland must also be kept moist with PBS during the procedure to avoid tissue damage.

When sectioning, detecting the presence of the small portion of adrenal tissue in the sections can be challenging due to tissue hypopigmentation after the processing step. A microscope is very helpful during the sectioning for discerning the tissue from the wax.

Immunostaining on paraffin-embedded sections is a valuable technique for immunolabeling proteins of interest while preserving the morphology of the tissue. The method presented here is based upon fluorescence detection and, while it is particularly functional for studies employing multiple primary antibodies, the fluorescence signal is light-sensitive and can be easily lost or weakened if the slides are not handled properly (*i.e.,* prolonged exposure to the microscope light or unnecessary exposure to ambient light). Moreover, we can notice a decline of the quality of the fluorescence signal itself over time. This problem can be avoided by using chromogenic detection, which is photostable and can be visualized for many years. This method, however, lacks the advantages of the fluorescence detection, such as higher labeling precision and simultaneous labeling of several proteins in one study.

Paraffin embedding is a convenient method to process and store multiple tissue samples. However, the tissue processing for paraffin embedding itself is not suitable for imaging of fluorescent reporters endogenously expressed in some transgenic animals without the use of a specific antibody targeting the reporter. Cryopreservation is a method to avoid degradation of the fluorescent protein and allow its direct visualization under a microscope. Avoiding the use of an extra antibody can represent an advantage. On the other hand, cryopreservation can also be limiting since it affects tissue morphology; it also requires different equipment for sectioning, and the storage of slides and tissue blocks is possible in freezers only.

One major limitation of imaging a sectioned tissue is the ability to obtain images of structural components at high spatial resolutions. Tissue composition, in fact, is a major variable in determining the quality of the imaging since it influences light penetration and can lead to poor resolution. The adrenal gland is a tissue rich in lipids that cause light scattering[22]. In the brain, which also has a high lipid content, tissue-clearing techniques such as CLARITY, BABB, iDISCO, and 3DISCO have been developed to improve tissue visualization, allowing for better imaging and 3D tissue reconstruction[23]. These techniques are providing researchers high quality imaging data, and are being adapted to a various range of tissues, and, in the future, we hope to employ these methods for adrenal imaging as well.

Disclosures

The authors have nothing to disclose.

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