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Examining the Endogenous Antioxidant Response Through Immunofluorescent Analysis of Nrf2 in Tissue

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Abstract

As organisms designed to depend upon oxygen to sustain life, humans are necessarily and continually exposed to damaging oxidizing agents. As a vital protective measure, oxygen-dependent organisms have developed a highly evolutionarily conserved mechanism for preventing oxidative stress. NF-E2 (nuclear factor (erythroid-derived 2))-related factor-2 (Nrf2) is the primary regulator of this endogenous antioxidant response. Many diseases that plague human society, ranging from various cancers to neurodegenerative diseases, have oxidative stress as a component of their etiology, and thus, much disease research has focused on Nrf2, both as a potential point of biological failure and as a promising therapeutic target. As a transcription factor, Nrf2 is active only when it is nuclear, and is regulated largely by its subcellular distribution. Thus, Nrf2 protein levels and subcellular localization are both key pieces of information when studying the endogenous antioxidant response. Immunofluorescent analysis (IFA) of Nrf2 in human tissue is a particularly powerful tool in the study of Nrf2 in disease, because it allows examination of both of these regulatory mechanisms that modulate Nrf2 activity.

Keywords

Nrf2; Immunofluorescence; Confocal microscopy; Endogenous antioxidant response

1. Introduction

Oxidative stress is a constant presence in the cells that make up animal life forms and, thus, inevitably plays a significant role in the pathogenesis of many diseases. To combat this omnipresent oxidative stress, animal cells have evolved an endogenous antioxidant response. Nuclear factor (erythroid-derived 2) (NF-E2)-related factor 2 (Nrf2), is the key regulator of antioxidant response genes, making it an essential player in this endogenous cellular antioxidant response ^{1, 2, 3, 4, 5, 6, 7}). Nrf2 is a member of the Cap 'n' Collar family of transcription factors, which includes Nrf1, Nrf3, p45, Bach1, and Bach2 ²). Nrf2 binds the antioxidant responsive element (ARE), also known as the electrophile response element (EpRE)), a *cis*-acting regulatory element found in the 5'-flanking region of genes encoding enzymes for antioxidant production and of other Nrf2 target genes ^{2, 3, 8}). Genes regulated by Nrf2 include heme oxygenase-1 (HO-1), ubiquitin/PKC- ζ -interacting protein A170, glutathione S-transferases (GST), NAD(P)H quinone oxidoreductase (NQO1), peroxiredoxin 1, catalase, glutathione peroxidase, superoxide dismutase (SOD), and thioredoxin ^{1, 4, 9, 10, 11, 12}). Additionally, Nrf2 regulates the expression of scavenger receptors, chaperone proteins, proteasome proteins, and transcriptional regulators ^{9, 11}).

Nrf2 is largely regulated through the control of its subcellular distribution, rather than through its induction, evidenced by the fact that Nrf2 mRNA levels remain relatively constant throughout treatment with oxidative stressors^{13, 14}). Subcellular localization of Nrf2 is controlled by the actin-binding, cytoplasmic Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1), which binds the N-terminal Neh2 [Nrf2-ECH homology 2] domain of Nrf2, tethering it to the cytoplasm^{2, 14}) (Fig. 18.1). In addition to regulating Nrf2 subcellular localization, Keap1 also regulates Nrf2 protein levels, by mediating proteasomal degradation of Nrf2. Keap1 binds the ubiquitin ligase, Cullin 3 (Cul3)¹⁵). In effect, Keap1 acts as a bridge between Cul3 and Nrf2, resulting in rapid Nrf2 degradation^{16, 17}). As shown in Fig. 18.1, in the presence of mediators of oxidative stress, such as reactive oxygen species (ROS), modification of Keap1 cysteines and phosphorylation of Nrf2 act cooperatively as regulatory mechanisms allowing Keap1 release of Nrf2, and consequently, nuclear translocation of Nrf2^{2, 18, 19, 20, 21, 22, 23, 24, 25}). This system of regulatory mechanisms provides precise control of Nrf2 activation.

While Nrf2 subcellular localization is a crucial determining factor of Nrf2 activity, several additional mechanisms modulate Nrf2 activity. Nrf2 DNA binding and transactivation both depend upon Nrf2 heterodimerization with small Maf proteins^{1, 26}). In addition to small Mafs, studies have suggested that c-Jun and activating transcription factor 4 (ATF4) also form heterodimers with Nrf2 and augment its transactivation activity^{27, 28}). Further, Nrf2 has two transcriptional activation domains. These domains bind CBP (cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) binding protein) or p300, which then mediate transcriptional activation of Nrf2 target genes^{29, 30}). Synergistic activity of simultaneous CBP/p300 binding at both Nrf2 transactivation domains may explain the large potency of Nrf2 transactivation activity as compared with that of other CNC transcription factors^{9, 29}).

The exquisite control over Nrf2 function (Fig. 18.1) implies an extreme importance of precise Nrf2 activity, and also provides a large number of potential points of malfunction and targets for therapeutic strategies in disease states affected by oxidative stress. Because Nrf2 is regulated by control of both its subcellular localization and its protein level, immunofluorescent analysis (IFA) is a particularly powerful tool for examining the endogenous antioxidant response as controlled by this transcription factor. In particular, IFA in human tissue allows study of the role Nrf2 plays in human disease. However, because Nrf2 is expressed at relatively low levels, particularly in certain tissues, such as brain, IFA of this protein is not straightforward. Further, IFA is only scientifically useful for semiquantitative analysis of protein levels and subcellular localization when it is performed with certain considerations to allow for distinction between slight variations in protein level and when it is analyzed with confocal laser microscopy.

2. Materials

2.1. Equipment

1. Microtome.
2. Biorad Radiance 2100 laser confocal microscope equipped with Argon, Green He/Ne, Red Diode, and Blue Diode lasers (Biorad, Hercules, CA, USA), as described previously³¹).

2.2. Reagents

1. Histoclear (National Diagnostics, Atlanta, GA, USA).

2. Ethanol (200 proof, Absolute, Anhydrous, ACS/USP Grade) in deionized H₂O (dI H₂O) at 70%, 90%, and 95%, and 100% ethanol.
3. A stable solution of 30% H₂O₂ (Sigma-Aldrich, St. Louis, MO, USA) is mixed with methanol (Thermo Fisher Scientific, Waltham, MA, USA) to a final concentration of 3% H₂O₂.
4. 10X Target Retrieval Solution (Dako, Carpinteria, CA, USA) in dI H₂O to its 1X concentration.
5. Phosphate buffered saline (PBS) (ScyTek Laboratories, Inc., Logan, UT, USA) purchased at 25X and diluted in dI H₂O to its final 1X concentration.
6. Normal goat serum (Chemicon (Millipore), Billerica, MA, USA) diluted in PBS to a final concentration of 5%.
7. Super Pap Pen (Ted Pella, Inc., Redding, CA, USA)
8. Normal Antibody Diluent (NAD) (ScyTek Laboratories, Inc., Logan, UT, USA).
9. Tyramide Signal Amplification (TSA Biotin System) (TSA Kit) (Perkin Elmer, Waltham, MA, USA).
10. Streptavidin FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).
11. Nucleic acid stains: DAPI (Molecular Probes), propidium iodide (Sigma-Aldrich), or Hoechst (Thermo Fisher Scientific) diluted in NAD according to manufacturer's recommendations.
12. RNase A (Roche Pharmaceuticals, Nutley, NJ, USA) diluted in NAD according to manufacturer's recommendation.
13. Citifluor AF1 mounting media (Citifluor, Ltd., London, UK).
14. Nail polish (any commercially available variety).
15. Primary antibodies: Nrf2 C-20, Nrf2 H-300 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Nrf2 MO1, Nrf2 MO3 (Abnova Corporation, Taipei City, Taiwan).
16. Secondary Antibodies (Jackson ImmunoResearch Laboratories Inc.): biotin-conjugated goat anti-rabbit antibody, biotin-conjugated goat anti-mouse antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody, FITC-conjugated goat anti-mouse antibody.

2.3. Supplies

1. MetaMorph 6.0 image analysis software (Universal Imaging, Inc, Downingtown, PA, USA).

3. Methods

When the endogenous antioxidant response is active, the overall protein levels of Nrf2 increase and its subcellular localization is altered. Given the importance of these two regulatory mechanisms in Nrf2 activation, immunofluorescent analysis (IFA) is an especially powerful tool for the examination of Nrf2-mediated endogenous antioxidant activity in human tissues. However, Nrf2 is a relatively scarce protein, particularly in certain types of tissue, such as brain. Thus, to successfully visualize this protein using immunofluorescence, the signal must be amplified using tyramide signal amplification (TSA). This amplification method utilizes the enzymatic activity of horseradish peroxidase (HRP), which induces activation of short-

lived, but highly reactive tyramide, which then covalently attaches to nucleophilic residues near the site to which the HRP is tethered. To start, Nrf2 is recognized by a primary antibody directed against it, which is then recognized by a secondary antibody that is conjugated to biotin. The biotin then binds HRP-conjugated streptavidin with high affinity, affixing the HRP in close vicinity to Nrf2 proteins. Then, biotinylated tyramide is added, and subsequently activated by the HRP causing it to covalently bind to nearby electrophilic residues. Finally, fluorescein isothiocyanate (FITC)-conjugated streptavidin binds to the biotinylated tyramide, allowing amplified visualization of Nrf2.

In addition to allowing visualization of scarce proteins, TSA also allows more flexibility in the choice of primary antibodies that can be used to triple- and quadruple-label tissue. Specifically, when TSA is not used, only one polyclonal antibody made in each type of animal may be used (e.g. only one rabbit antibody, not two, may be used in any given sample), or in the case of monoclonal antibodies, only one of each type of antibody isotype may be used. However, when using TSA, primary antibodies can be used at such a low dilution that they are not visible without the use of TSA. Thus, after TSA is used to amplify one antibody, even though a second secondary will recognize both primary antibodies made in the same animal, the TSA-amplified antibody will be such a small amount that it will not contribute to the signal visualized with the second secondary.

While TSA is a powerful tool for the visualization of Nrf2, it must be used appropriately if it is to allow for discrimination between protein levels in different experimental conditions (e.g. disease versus normal). Specifically, the appropriate dilution factor for the primary antibody must be empirically determined prior to data generation. Further, confocal laser microscopy must be used to visualize fluorescent staining of Nrf2, when attempting to accurately discern subcellular localization of this protein.

3.1. Immunofluorescent labeling in paraffin-embedded tissue

1. Slice paraffin-embedded tissue into 7 μ m sections using a microtome. Mount individual sections on (+) charged slides and allow them to dry, and, thus, they become fixed to the slide.
2. Heat paraffin-embedded sections to 55°C overnight. If necessary, this step can be shortened, but slides must be heated for at least 30 minutes. Then, deparaffinize slides by 3 incubations in Histoclear for 10 minutes each (*see Note 1*).
3. Rehydrate sections as follows: 2 incubations for 10 minutes each in 100% alcohol followed by 5-minute incubations in each of 95% alcohol, 90% alcohol, 70% alcohol, and deionized H₂O.
4. Incubate slides for 30 minutes in a solution of 3% H₂O₂ in methanol to quench endogenous peroxidase activity in the tissue, followed by a 5-minute wash in deionized H₂O.
5. Perform antigen unmasking by immersing slides for 1 hour in Target Retrieval Solution (DAKO) preheated to 95°C (*see Note 1*). After a 1-hour incubation in the Target Retrieval Solution at 95°C, remove the Coplan jar containing the slides from the water bath to the benchtop, and allow it to cool gradually to room temperature. After cooling, wash slides three times in PBS for 5 minutes each.
6. To minimize nonspecific binding of secondary antibodies, block slides by incubating them for 1 hour in a phosphate-buffered saline (PBS)-based solution of 10% normal serum of the animal in which the secondary antibody was produced. This incubation is done at room temperature. Unless otherwise indicated, all incubations and washes

should be conducted at room temperature. After 1 hour of blocking, wash slides three times in PBS for 5 minutes each.

7. Remove slides from the PBS wash one at a time, dry the portions of the slide that have no tissue with a paper towel and encircle the tissue with the hydrophobic marking of a Pap Pen (*see Note 2*). Apply Nrf2 primary antibody to the area enclosed by the Pap Pen mark and incubate slides overnight at 4°C. Antibody should be diluted in Normal Antibody Diluent (NAD). To allow for discrimination between the levels of protein in different experimental conditions (e.g. diseased versus normal tissue), determination of the correct primary antibody dilution is critical, and must be empirically determined prior to use of the antibody for data generation, (*see Section 3.2* for a detailed procedure for empirically optimizing primary antibody dilution.) After overnight incubation, wash slides three times in PBS for 5 minutes each.
8. This step begins the TSA amplification process. Incubate slides for 30 minutes (*see Note 3*) in biotin-conjugated secondary antibody directed against immune fragments of the animal in which the primary antibody was made (e.g. use an antibody directed against rabbit IgG for a primary antibody made in a rabbit). For all secondary antibodies used throughout the course of the protocol, use antibodies made in the animal against which the tissue was blocked in step 6. Secondary antibodies should be diluted in NAD, following the manufacturer's recommendation (*see Note 4*). Following the 30-minute incubation, wash slides three times in PBS for 5 minutes each.
9. Incubate slides first in TNB (Tris NaCl Blocking)(TSA kit) Buffer for 30 minutes, then in horseradish peroxidase (HRP)-conjugated Streptavidin (TSA kit) diluted in TNB at a dilution factor of 1:400 for 30-minutes. Following these incubations, wash slides three times in PBS for 5 minutes each.
10. Incubate slides for 30 minutes in biotinylated tyramide, diluted 1:100 in amplification diluent (TSA kit). Following this incubation, wash slides three times in PBS for 5 minutes each (*see Note 5*).
11. Incubate slides for 30 minutes in Streptavidin conjugated to fluorescein isothiocyanate (FITC) or another fluorescent molecule whose excitation and emission frequencies are different from those of the nucleic acid markers to be used (e.g. DAPI or propidium iodide). Following this incubation, wash slides three times in PBS for 5 minutes each (*see Note 6*).
12. Incubate slides in second primary antibody for 1 hour at 37°C, 2 hours at room temperature, or overnight at 4°C (*see Note 7*). The second primary antibody may be a cell-type specific marker, used to allow determination of cell-type specific Nrf2 staining, or it may be another experimental protein of interest. However, the recognized protein must be sufficiently abundant so that its visualization does not require TSA amplification, as only one course of TSA amplification may be used in any staining protocol. Following this incubation, wash slides three times in PBS for 5 minutes each.
13. Incubate slides for 30 minutes in the second secondary antibody, which should be conjugated to cyanine 3 or another fluorescent molecule whose excitation and emission frequencies are different from the fluorescent molecule already used during TSA amplification and from the nucleic acid markers to be used. This antibody should be directed against immune fragments of the animal in which the second primary antibody was made and should be diluted in NAD, following the manufacturer's recommendation. Following the 30-minute incubation, wash slides three times in PBS for 5 minutes each.

14. Steps 12 and 13 can be repeated for a third or fourth primary antibody. Please note, no two primary antibodies may be used that were made in the same type of animal, except for the host animal of the antibody amplified using TSA, which may be used twice (*see Section 3.3*⁽³⁾ for necessary controls if this is to be the case). Additionally, each primary antibody must be visualized using a fluorescent molecule with distinct excitation and emission frequencies from others used in the same staining protocol.
15. If desired, incubate slides in a nucleic acid stain to label cell nuclei (*see Notes 8 and 9*). Dilute stain according to manufacturer's recommendation. If propidium iodide or Hoechst stains are to be used, slides must first be incubated in RNase A, diluted according to manufacturer's recommendation, for at least 30 minutes at room temperature.
16. Coverslip slide using three drops of Citifluor mounting medium (*see Note 10*). Seal the edges of the coverslip with nail polish to prevent leakage of the mounting medium and the resultant drying of the tissue.

3.2. Empirically optimizing primary antibody dilution

1. To allow for discrimination between the levels of protein in different experimental conditions (e.g. diseased versus normal tissue), use of the correct primary antibody dilution is critical, and must be empirically determined prior to use of the antibody for data generation. If too much antibody is used, the signal becomes saturated and any differences between experimental conditions are masked and cannot be seen, thereby resulting in a type II error (false positive) (*see Note 11*).
2. To empirically determine the appropriate primary antibody dilution, test different dilutions in one case from each experimental condition (e.g. disease versus normal), which will allow discernment of differences in levels of the target protein. For this process, it may be possible to avoid use of tissue that is highly experimentally valuable, in favor of tissue that is relevant but not as valuable. For example, when studying Alzheimer's disease, a disease that greatly affects hippocampal brain tissue, determination of appropriate primary antibody dilutions can often be conducted in a brain region that is more abundant, but still has pathology such as the anterior cingulate cortex, using both normal and diseased tissue.
3. Determination of the ideal dilution may require several rounds of empirical testing. To start, use dilutions that span the range provided by the manufacturer, in a nonlinear distribution. For example, if the manufacturer recommends using a dilution between 1:100 and 1:1000, the testing dilutions should be 1:100, 1:300, and 1:1000. If the manufacturer does not provide recommendations and there are no previous publications using the antibody for IFA, start testing dilutions between 1:100 and 1:1000. Ideally, protein level differences will be visible with one of the dilutions used. However, if this is not the case, and if your lowest dilution factor does not show staining, then try lower dilution factors using a similar distribution scheme. Alternatively, if even the most dilute antibody results in very robust staining, then repeat the process using higher dilutions with a similar distribution (i.e. 1:1000, 1:3000, 1:10000). When one dilution shows potential as the optimal dilution, one more test should be conducted using dilutions closer to the chosen dilution. *See Fig. 18.2 for an example protocol scheme.*

3.3. Controls

1. During empirical determination of the appropriate dilution factor for the primary antibody, two controls must be performed. Both of these controls need only be performed once for a given type of tissue (species and tissue type) and a given

antibody. However, when a new lot of any antibody (primary or secondary) is purchased, these controls should be rerun, to ensure that the antibody has not changed from lot to lot.

2. The first control is conducted to ensure that the secondary antibody does not cause nonspecific background staining and must be conducted each time a new secondary antibody is used, regardless of the primary antibody being used. Prepare one slide that is not incubated in primary antibody. Instead, this slide should be incubated overnight in NAD as the other slides are incubating in primary antibody (*see Section 3.1*⁽⁷⁾). Following this step, treat this control slide exactly as the other slides are treated, following each step of the protocol. This control slide should show no staining.
3. The second control acts to ensure that there is no visible Nrf2 signal if the primary antibody signal is not amplified using TSA. This control must be performed every time a new primary antibody is used with TSA, regardless of the secondary antibody being used, and is particularly important if a second primary antibody made in the same animal will be used (e.g. if using both a rabbit Nrf2 antibody and a rabbit cell-type marker antibody in the same slides). This control slide is prepared by skipping the TSA process. Thus, to prepare this slide, at step 8 (*see Section 3.1*⁽⁸⁾), which starts the TSA amplification, rather than using the biotin-conjugated secondary antibody, incubate your control slide with a secondary antibody conjugated to the same fluorescent molecule as that conjugated to the streptavidin in the final TSA step (*see Section 3.1*⁽¹¹⁾). Do NOT treat this slide with the remaining TSA steps, but rather, continue with the protocol starting from incubation with the second primary (*see Section 3.1*⁽¹²⁾). This control slide should show no staining.

3.4. Confocal laser microscopy image capture

1. Images should be captured using confocal laser microscopy to allow determination of Nrf2 subcellular localization, specifically whether it is in the nucleus or the cytoplasm. When choosing which fluorescent molecules to use for visualization of antibody staining, consider the lasers included in available confocal microscopes. Collect 5–10 images from each tissue section. Images should be collected randomly from within the regions of interest in each section (e.g. in cortical brain tissue consider gray versus white matter when collecting images) and in a researcher-blinded manner. Collect all images at uniform settings to allow comparisons across samples.

3.5. Post-acquisitional analysis

1. Perform post-acquisition analysis for immunofluorescent staining using MetaMorph 6.0 image analysis software (*see Section 2.3*⁽¹⁾). To determine total Nrf2 protein levels, measure integrated pixel intensity for Nrf2 per image, where the integrated pixel intensity is defined as total pixel intensity per image times the area of pixels positive for the signal. For colocalization of Nrf2 with specific cell types in each image, examine integrated pixel intensity for Nrf2 that is overlapping with the pixels positive for your specific cell-type marker. Often when determining Nrf2 colocalization with specific cell types, it is necessary to normalize the values to the area of pixels positive for the specific phenotypic marker to account for variations in phenotypic marker expression in different experimental conditions. Additionally, Nrf2 subcellular localization can be examined in a similar manner by determining Nrf2 colocalization with unclear markers, such as propidium iodide.

4. Results

1. AD is a region-specific neurodegenerative disease, affecting in particular the hippocampus and various parts of the cortex. Examination of Nrf2 in hippocampal autopsy tissue from AD patients reveals that Nrf2 subcellular localization is altered as compared with control autopsy hippocampal tissue (³²). In this study by Ramsey *et al.*, phenotypic cell-type specific markers and nucleic acid stains demonstrate that Nrf2 is present in both neurons and astrocytes, and allow discrimination of different Nrf2 subcellular staining patterns in these two cell types (Figure 2 from (³²)). Specifically, the findings presented by Ramsey *et al.* show that in normal hippocampal CA1 neurons, Nrf2 is predominantly nuclear, while in AD hippocampal CA1 neurons, it is predominantly cytoplasmic, and therefore not transcriptionally active. These findings suggest that Nrf2 is unable to initiate the endogenous antioxidant response in neurons in the hippocampi of AD patients, and may thereby contribute to pathogenesis of this disease that tragically affects so many lives.
2. The protocol outlined in this chapter can be extended to examination of proteins other than Nrf2. As can be seen in Lindl *et al.* (³³), the endoplasmic reticulum (ER) stress response protein, Binding Protein (BiP), can be visualized using IFA with TSA in human midfrontal cortical autopsy tissue from HAD patients. In this tissue, BiP total protein levels were quantified using Metamorph software and were seen to increase in gray matter. Additionally, astrocytic BiP increased as determined using Metamorph software to analyze BiP staining that colocalized with astrocytic marker, GFAP staining. These findings demonstrate that the ER stress response is activated in the midfrontal cortical gray matter in HAD autopsy tissue.

5. Notes

1. The antigen-unmasking step (*see* **Section 3.1**(⁵)) is usually done in a Coplan jar, which will crack if heated quickly. Thus, it is prudent to prepare the Target Retrieval Solution in a Coplan jar while the slides are in the first step of HistoClear, and place the jar in a water bath set to 37°C and allow the Target Retrieval Solution to heat as the water bath heats up to 95°C.
2. When applying Pap Pen, be careful not to mark over the tissue and do not allow the tissue to dry out.
3. As a general rule, all 30-minute incubations should be limited to 30 minutes. Specifically: do not allow slides to incubate in secondary antibodies for much longer than 30 minutes, as this may increase nonspecific binding of the secondary anti-body; do not allow slides to incubate in Streptavidin-HRP or -FITC for much longer than 30 minutes (*see* **Section 3.1**(⁹)), as this may increase background staining; finally, **it is critical** that slides are not allowed to incubate in biotinyl-tyramide for longer than 30 minutes (*see* **Section 3.1**(¹⁰)), as this step utilizes an enzymatic activity and, thus, additional incubation time may significantly increase staining, resulting in an over-amplification of both specific and nonspecific antibody signal. Two exceptions to this general rule: 1) endogenous peroxidase quenching (*see* **Section 3.1**(⁴)), which can be extended to several hours and 2) the TNB incubation (*see* **Section 3.1**(⁹)), which can be greatly extended, but care should be taken that TNB does not remain on slides for a length of time that would allow contamination of the buffer.
4. Occasionally, the manufacturers, recommended dilutions for secondary antibodies are not optimal and result in high non-specific or background staining. If this is the case, apply the steps outlined in **Section 3.1**(¹) for optimization of the primary antibody dilution to optimization of the secondary antibody dilution.

5. After step 10 (*see* **Section 3.1**⁽¹⁰⁾), slides should be kept in the dark as much as possible. Changing of solutions can be done in a light room, but during washes and incubations, slides should be kept in a dark place.
6. If the Nrf2 antibody is the only primary antibody that will be used, proceed directly from step 11 to step 16 (*see* **Sections 3.1**⁽¹¹⁾–**3.1**⁽¹⁶⁾).
7. Incubations in primary antibody should be carried out for 1 hour at 37°C, 2 hours at room temperature, or overnight at 4°C. Ideally, slides should be incubated in the Nrf2 antibody overnight at 4°C. Further, any additional experimental proteins of interest should be incubated overnight at 4°C, ideally. However, antibodies to cell-type specific markers often work well in any of the three incubation conditions, and do not result in nonspecific background staining or weak specific staining. In particular, antibodies to microtubule-associated protein 2 (MAP2) and glial fibrillary-associated protein (GFAP) are known to work at any of the incubation conditions.
8. While all nucleic acid stains thoroughly mark astrocytic and oligodendrocyte nuclei, propidium iodide most clearly marks neuronal nuclei.
9. To save time, RNase A and nucleic acid stains can be included in other incubations; in particular, secondary antibody incubations, since they utilize the same conditions, are particularly convenient. However, please note the RNase A step *must be completed prior* to adding propidium iodide or Hoechst stain.
10. When coverslipping, avoid trapping air bubbles on top of the tissue in the Citifluor, as this will distort the visualization of the tissue. To prevent air bubbles, roll the coverslip onto the tissue from one end of the slide, rather than dropping it directly onto the slide.
11. The ideal antibody dilution will result in a signal just stronger than that required to visualize the protein of interest, thus avoiding saturation of the signal.
12. This protocol can be used for immunohistochemistry with some slight modification. Specifically, rather than using fluorescent-tagged streptavidin in the final TSA step (*see* **Section 3.1**⁽¹¹⁾), use Strep-HRP as used in step 9 (*see* **Section 3.1**⁽⁹⁾). To visualize Nrf2 staining in this case, use a kit such as Vector Red (Vector Laboratories, Burlingame, CA, USA) according to manufacturer's recommendation. Then, dehydrate slides by immersion for 5 minutes each in dI H₂O, 70% ethanol, 90% ethanol, 95% ethanol, and two times in 100% ethanol. Finally, coverslip the slides using Permount. Keep in mind that immunohistochemistry does not have the quantitative power of IFA and that it does not allow for the use of cell type markers to distinguish Nrf2 expression in different cell types.
13. This protocol can be used for IFA in tissue culture with some slight modification. First, wash cells three times in PBS for 5 minutes each and then two times for 10 minutes each in PBS with 0.1% Tween-20. Then, fix cells using a 30-minute incubation period in 4% paraformaldehyde/2% sucrose in PBS. Skip the steps required for deparaffinization, and proceed directly to blocking. However, include 0.1% Triton-X 100 in the blocking solution, to ensure the cells are fully permeabilized.

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Fig. 18.1.
Schematic for Nrf2 regulation and function.

Example:

1:100

1:300

1:1000

Fig. 18.2.
Optimization of primary antibody dilution.