Video Article

Imaging of Estrogen Receptor-α in Rat Pial Arterioles using a Digital Immunofluorescent Microscope

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Abstract

Many of estrogen's effects on vascular reactivity are mediated through interaction with estrogen receptors [1, 2, 3]. Although two sub-types exist (estrogen receptor -α and β),estrogen receptor-α has been identified in both the smooth muscle and in endothelial cells of pial arterial segments using fluorescent staining combined with confocal laser scanning microscopy [4]. Furthermore, ER-α is located in the nuclei and in the cytoplasm of rat basilar arteries [5]. The receptors are abundant and fluoresce brightly, but clear visualization of discrete groups of receptors is difficult likely due to the numbers located in many cell layers of pial vessel segments. Additionally, many reports using immunohistochemical techniques paired with confocal microscopy poorly detail the requirements critical for reproduction of experiments [6]. Our purpose for this article is to describe a simple technique to optimize the staining and visualization of ER-α using cross-sectional slices of pial arterioles obtain from female rat brains. We first perfuse rats with Evans blue dye to easily identify surface pial arteries which we isolate under a dissecting microscope. Use of a cryostat to slice 8 μm cross sections of the arteries allows us to obtain thin vessel sections so that different vessel planes are more clearly visualized. Cutting across the vessel rather than use of a small vessel segment has the advantage of easier viewing of the endothelial and smooth muscle layers. In addition, use of a digital immunofluorescent microscope with extended depth software produces clear images of ten to twelve different vessel planes and is less costly than use of a confocal laser scanning microscope.

Video Link

The video component of this article can be found at https://www.jove.com/video/3203/

Protocol

1. Isolation and Preparation of Pial Arteries

1. While the rat is anesthetized insert and secure a catheter into an artery and perfuse with 1% Evans blue in 1X PBS. Pial vessels stain well with the Evans blue dye making them easier to isolate.
2. Extract the brain and store at -70°C until needed. Optimally, brains should be stored no longer than 2 months.
3. Using a dissecting microscope remove pial arterioles (average diameter 35-50 μm) from the surface of the brain . Carefully pull off all the blue-stained arterioles from dorsal and lateral the surface of the cortex using fine- tipped forceps. Remove excess adherent cortical tissue before placing in the fixative.
4. Fix the harvested arterioles in 2% cold paraformaldehyde in 0.1M PBS for 30 min.
5. To prepare the arterioles for sectioning pour embedding medium on the freezing disc/chuck (set at -20°C).
6. Place arteriole section flat on the chuck and wait for it to freeze. Firmly place the vessel up-right on the cryostat freezing disc on embedding media with the tip of the artery facing you. Hold it with tweezers until it is solidly frozen.
7. Place the freezing disc with the vessel into the cryostat head and cut 8 μm pial arteriole rings with the cryostat.
8. Mount the arteriole sections on chromium-potassium-gelatin subbed slides.
9. Store the prepared slides in a slide box in the refrigerator at 4°C overnight.

2. ER-α Immunofluorescent Staining

Day 1

1. Remove the slides from the refrigerator; bring each to room temperature.
2. To wash the slides pour 1-1.5 ml 0.1M PBS (enough to cover all the sections) let sit for 10 minutes, drain and repeat procedure twice more. Each edge of the slide has a hydrophobic marker. Consequently, the liquid remains on the surface of the slide. With each washing the liquid is poured out and replaced with fresh 0.1 M PBS.
3. Incubate slides in 1 ml of 50mM ammonium chloride for 30 min at room temperature to reduce endogenous fluorescence.

4. Wash the slides in 0.1M PBS 3x10 min. as described in 2.2
5. Block slides in 0.1% Triton-X 100 plus 1% normal goat serum (NGS) in PBS for 30 min. to reduce non- specific binding.
6. Incubate samples with the primary antibody (rabbit polyclonal anti-ER-α; 1:500) in PBS + 0.1% Triton-X + 1% NGS overnight at 4°C.

Day 2

7. Remove the slides from the refrigerator; bring each to room temperature.
8. Wash the slides in 0.1M PBS 3x10 min. as described in 2.2
9. Incubate with Oregon Green 488 secondary anti-rabbit in 1% NGS +0.1%Triton-X100 + PBS for 2 hour in the dark at room temperature. From this step, the next steps must be done in the dark.
10. Wash the slides in 0.1M PBS 3x10 min. as described in 2.2
11. Under a ventilated hood, place 1 drop of 4', 6-diamidino-2-phenylindole (DAPI) plus mounting media on the vessels and then place a cover slip over the sample.
12. Apply clear nail polish to seal the edges of the cover slip. Do not move the slides until completely dry, which takes approximately 24 hours.

3. Digital Fluorescence Imaging

1. For imaging ER-α in the pial vessel slice we use a Nikon Eclipse 80i digital fluorescent microscope with filters for 3 colors (blue, green and red) equipped with a digital camera.
2. Insert the prepared slides with the stained pial vessel segments under the microscope and adjust to visualize the fluorescently labeled areas of interest. Start at x100 magnification then increase to x600 magnification.
3. Use the camera to capture images in DAPI (blue) and FITC (green) channels for cell nuclei (DAPI) and ER-α (Oregon Green) using Nikon Extended Depth Focus software to convert multiple views of vessel segments into 2-D images.

4. Representative Results:

We localized estrogen receptor-α in pial arterial vessels using fluorescent probes and optimized our visualization of the receptor using digital fluorescence microscopy. To detect the presence of the receptor, a rabbit polyclonal primary antibody and an Oregon Green 488 labeled secondary antibody was used to image the receptors in pial arteries isolated from the surface of the brain. Also, a nuclear stain (DAPI) was used to identify the cell nuclei and determine if we could detect receptors located in the nucleus since ER-α has been reported to be present in the cell cytosol and the nucleus. In addition, we conducted confirmatory experiments to validate the specificity of the primary antibody and to verify binding of the secondary to the primary antibody.

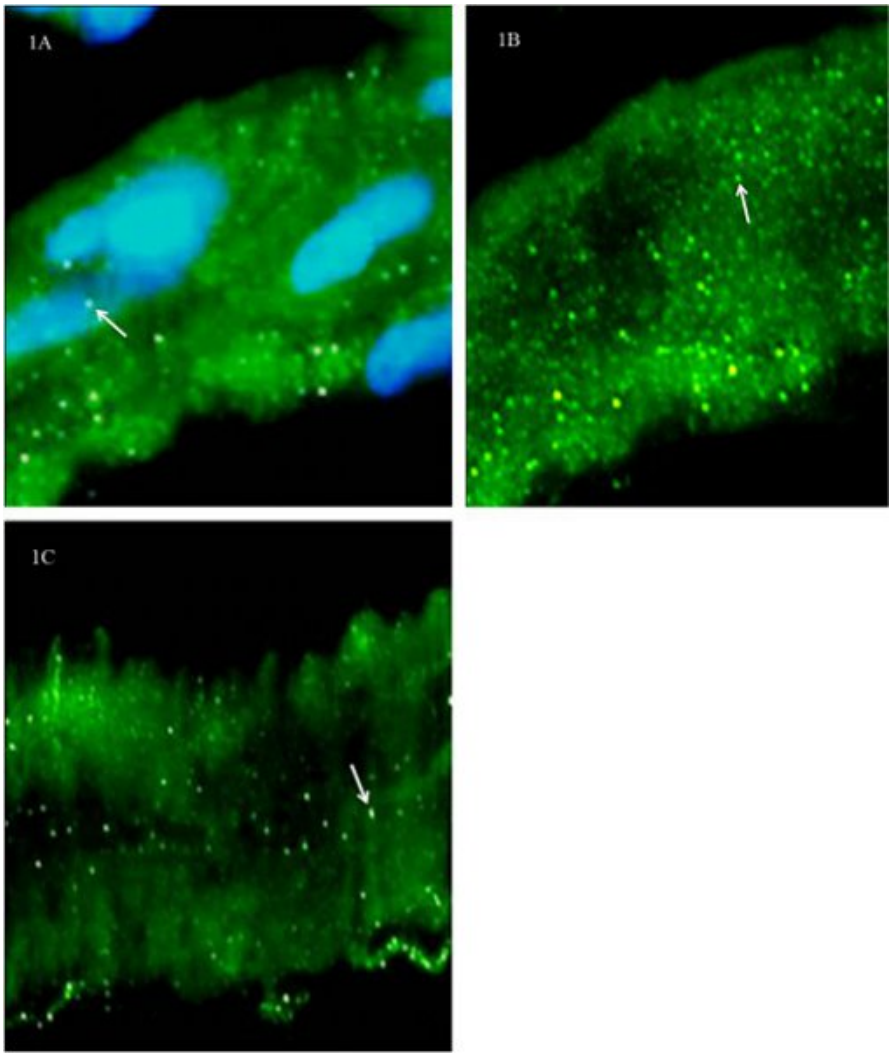

Figure 1. Immunofluorescent staining for ER- α (green) and counterstaining with DAPI (blue) in a pial artery ring isolated from female rat brain. Fig 1A shows the merged images suggesting the presence of some intranuclear estrogen receptor-a (arrow). Fig 1B and 1C are the focused image of ER- α which is a combination of Z-stack images. Images were taken at x600 magnification and are from the cut ring of the same vessel in different planes. Arrows indicate estrogen receptor- α.

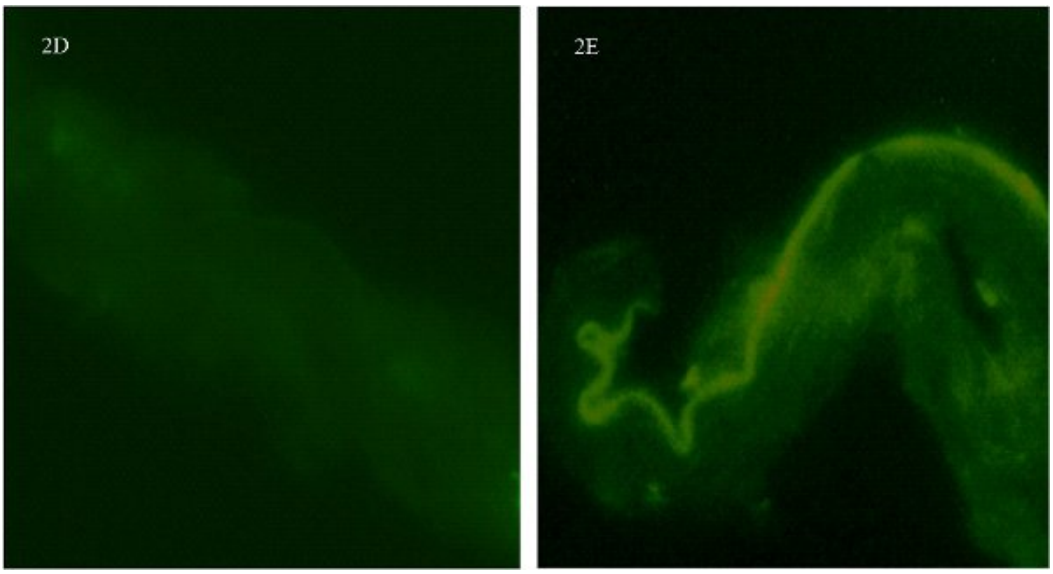

Figure 2. Immunofluorescent staining for control groups in a pial artery ring isolated from female rat brain. Fig 2D shows the vessel without primary antibody. Fig 2E shows the vessel without the secondary antibody (note distinct autofluorescence along endothelial side). Images were taken at x600 magnification and are from the cut ring of the same vessel in different planes.

Discussion

Previously, we showed that after transient global ischemic brain injury the pial artery capacity to change diameter in response to both vasodilators [7, 8] and vasoconstrictors [9] is markedly depressed in young ovariectomized rats. Chronic estrogen repletion restores vasomotor responses [7-9]. Further, delayed estrogen replacement reverses the beneficial effects of estrogen on pial artery vasodilatory capacity [10] leading us to question whether long-term estrogen depletion affects ER-α density in the cerebrovasculature. We planned to use immunofluorescent labeling combined with western blotting to evaluate changes in ER-α expression in response to chronic estrogen depletion. Because we had some difficulty in visualization of discrete groups of receptors we modified the method that we demonstrate here. Therefore, our primary goal in this study was to first develop a relatively simple method to optimize the visualization of the receptors in pial arterioles. In agreement with several reports [6,11,12] we found it necessary to make adjustments for the type of tissue we were probing, the sample thickness, the distribution of ER-α in the vessel, as well as the degree of non-specific binding.

Our method is very effective to visualize the presence of ER-α in pial vessel slices. But as others have previously pointed out the successful use of these methods is highly dependent on the specificity and concentrations of the antibodies, the subcellular location of the proteins of interest, fixation and blocking protocols and tissue handling [12]. Prior to visualization of our vessel preparations we spent time to work out all these variables. We have highlighted how we needed to adjust some of our approach in this paper.

We set out to optimally visualize ER-α in pial arterioles and this presented a few challenges. First, the average size of a rat pial artery is between 35-50 μm making isolation and removal from the brain surface somewhat tricky. We found that perfusion with Evans blue dye prior to sacrifice makes dissection of the arteries easier. Evan's blue can affect fluorescence, but we feel that the advantage of using this dye outweigh its minor disadvantages. In our protocol the vessels are washed several times. This minimizes the residual dye in the vessels and reduces the possible effects of the dye on fluorescence. Another difficulty we encountered was that the thickness of the pial vessel segments made clear visualization of the receptors difficult. Additionally, we could not obtain particularly clear images probably due to the fact that ER-α was scattered throughout several cell layers. We tried working with longitudinal vessel slices (as others have), but due to the small pial size it was difficult to handle the vessels and prevent twisting of the vessels while mounting on slides even using a microscope. Finally, we successfully modified our technique by using a cryostat and cutting the vessel cross-wise in to 8 μm thick rings. The rings are easier to handle and several can be mounted on one slide.

Once we acquired the vessels we felt it necessary to test the effectiveness of first flash freezing the tissue then fixing the vessels as suggested by Danseshatalb and associates [11]. Although we found marginally less background immunofluorescence, we also noted that the receptors stained less intensely making visualization of the receptors more difficult. Consequently, we prefer to first to freeze the entire brain and store until needed (generally between 1-2 months). We then pull off the vessels as described and fix the vessels prior to slicing with the cryostat. as we demonstrate here.

One necessary step is to run controls with both the primary and the secondary antibody separately to determine specificity and background immunofluorescence. Estrogen receptor-α antibodies can be difficult to work with. In fact, we tried 3 different primary antibodies (1 monoclonal and 2 polyclonal) before we were satisfied with the success of our receptor staining. As part of our controls we first verified the specificity of the primary antibody by omission of the addition of the primary antibody. Figure 2D shows the vessel without primary antibody. There is no staining and just a little background signal. Since the primary antibody is polyclonal, there is some diffuse non-specific background staining. Secondly, we added the primary antibody, but not the secondary antibody (Fig. 2E). One can see a nice margin representing the autofluoresence along the endothelial borders of the vessels. This is consistent with the work of Dan and associates [5]. However, neither the small points of immunofluorescence that represent ER-α , nor the granular appearance reflecting the presence of numerous receptors can be detected without the combination of both the primary and secondary antibodies. We noted a distinct pattern of autofluoresence along the endothelial margins of the vessels. These independent tests for antibody specificity are important as they confirm the specificity of the antibody for ER-α and the secondary antibody binds to the primary.

In conclusion, as demonstrated by others [4, 5] brain blood vessels contain numerous ER-α sub-type receptors. In our hands, preparing 8μm cross sectional slices from isolated pial arteries enhances staining and visualization of the receptors. Traditional methods use confocal microscopy to examine the samples. Having the capacity to view serial optical sections from thick specimens is an important benefit of confocal microscopy. However, purchase of a good confocal microscope can be very expensive. By using Extended Depth (EDF) software, we found that could reduce our equipment costs. Using a fluorescent microscope with EDF we obtained clear images and are able to visualize pial arterial ER-α on several levels using Z-stacks. A significant benefit of the EDF software is that it permits image capture in a manner to effectively create a 3-D picture from the images. For laboratories that do not have access to a confocal microscope or the funds to purchase one a digital fluorescent microscope with appropriated software may be a practical and less costly option depending on the goals of the investigator.

Disclosures

The views expressed are those of the authors and do not reflect the official policy or position of the Uniformed Services University of the Health Sciences, the Department of Defense, or the United States government.

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