Staining the endoplasmic reticulum in combination with antibody staining.

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Abstract

The endoplasmic reticulum is a vital cellular organelle involved in protein production, trafficking and cell communication. The central role of the endoplasmic reticulum makes its visualisation relevant across a range of cell biology research fields. A number of dyes are available for imaging endoplasmic reticulum in live cells, however, imaging fixed cells has been far more problematic. The ability to stain the entire endoplasmic reticular structure in fixed cells provides greater insight into the interactions between specific proteins of interest and the endoplasmic reticulum. In this protocol we show that a commercial endoplasmic reticulum marker ReZolve-ER can be used to visualise the endoplasmic reticulum following standard immunofluorescence antibody labelling. This protocol enables researchers to gain insight into protein and endoplasmic reticulum interactions without requiring live-cell imaging capabilities, and enables visualisation of the complete endoplasmic reticulum organelle network in fixed cells.

Reagents

1. Poly-L-Lysine \ (if working with non-adherent cells) 2. Aqueous mounting media 3. 4 % Paraformaldehyde 4. Phosphate Buffered Saline \ (PBS) 5. Saponin 6. Tween 20 7. Primary antibody of choice 8. Appropriate secondary antibody \ (we recommend Alexa Fluor 647) 9. ReZolve-ER 10. DMSO

Equipment

1. #1.5 glass coverslips 2. Glass slides 3. Confocal microscope \ (Nikon A1+)

Procedure

****Cell Culture**** Use standard culture protocols for maintenance of cells. For adherent cells, seed on sterile coverslips for 24-48 hours. For non-adherent cells, cells can be fixed in suspension and attached to poly-l-lysine coated coverslips before antibody staining. For this protocol we used the adherent cell line PNT2 grown in RPMI media supplemented with 10 % fetal bovine serum. Cells were seeded at 1×10^5 cells / mL in 6 well plates and cultured for 48 hours. ****Fixation**** We recommend fixing with paraformaldehyde. Cold methanol fixation can be used, however this may reduce ER staining intensity. 1. Fix cells in 4% paraformaldehyde in PBS pH 7.4 for 10 minutes at room temperature. 2. Wash cells three times with PBS for five minutes each at room temperature. ****Permeabilisation**** 1. Incubate the samples for 15-25 minutes with 0.5% w/v saponin in PBS at room temperature. 2. Wash cells three times with PBS for five minutes each at room temperature. ****Blocking**** 1. Incubate cells with 1% BSA, in PBST \ (PBS+ 0.1% Tween 20) for 30 min at room temperature to block non-specific binding of the antibodies ****Antibody staining**** 1. Prepare primary antibody to required concentration in 1% BSA in PBST. For this example we use rabbit anti-Cytochrome-C \ (Abcam ab90529 0.6m/mL) at a concentration of 1 µg/ml \ (for localisation to mitochondria). 2. Incubate cells in the diluted primary antibody for one hour at room temperature or overnight at 4°C. 3. Remove primary antibody and wash the cells three times in PBS for five minutes each. 4. Prepare secondary antibody to required concentration in 1% BSA in PBST. For this

example we used goat anti-rabbit Alexa Fluor 647 at a 1/1000 dilution.* 5. Incubate cells with the secondary antibody for one hour at room temperature in the dark. 6. Aspirate the secondary antibody and wash cells three times with PBS for five minutes each. * CAUTION for best results select a secondary antibody with a fluorophore which is compatible with ReZolve-ER (ex. UV or 400 nm, em. 570 nm). We recommend a far-red fluorophore such as Alexa Fluor 647. Whichever fluorophore you select ensure that it is not excited at UV or 400 nm excitation. **Endoplasmic Reticulum Staining** 1. Prepare 10 mM stock solutions of ReZolve-ER by dissolving the powder in DMSO as instructed on the vial. 2. Prepare a staining solution by diluting the stock in PBS to 20 μ M (1/500 dilution). 3. Incubate cells with staining solution for 30 min at room temperature, in the dark. 4. Remove coverslips from the staining solution and mount in aqueous mounting media. For best results ensure some of the staining solution is transferred into the mounting media.* * CAUTION do not wash coverslips after ReZolve-ER staining. Washing will significantly reduce the fluorescence obtained from the staining. **Mounting and Imaging** 1. Once mounted adhere coverslips to the slide using quick-dry nail polish. 2. For best results, imaging by confocal microscopy is recommended. 3. For excitation of ReZolve-ER a UV, 385 nm or 400 nm light source can be utilized. For detection, a detector compatible with FITC is most appropriate, or if available a tuneable detector set to a detection range of 525 nm - 600 nm. 4. For antibody imaging use appropriate settings for the selected secondary antibody. For this example we have used a 647 nm excitation and a detector compatible with far-red fluorophores. 5. To avoid signal bleed through, sequential imaging is recommended.

Timing

Cell Culture 24 - 48 hours **Fixation** 25 minutes **Permeabilisation** 25 minutes **Blocking** 30 minutes **Antibody Staining** 2 hours 30 minutes **Endoplasmic Reticulum Staining** 30 minutes **Mounting and Imaging** 1 - 2 hours

Troubleshooting

Some troubleshooting may be required for different antibodies. To ensure antibody is maintaining localisation, perform antibody only staining in parallel to compare antibody distribution with and without ReZolve-ER staining. Adding a small amount of ReZolve-ER staining solution to mounting media can improve the endoplasmic reticulum stain achieved. To improve ReZolve-ER detection by confocal microscopy use slow collection speeds and high averaging (2 to 4).

Anticipated Results

Using this protocol the endoplasmic reticulum structure will be visible as a large network like structure throughout the cells, with greatest density in the perinuclear region (Figure 1A). Antibody staining will depend on your protein of interest, but should be unchanged by the presence of ReZolve-ER. In this example we have targeted the mitochondrial protein Cytochrome-C. As expected the protein is present throughout the cytoplasm (Figure 1b) and in close proximity to the endoplasmic reticulum, due to

association between the mitochondria and the endoplasmic reticulum \ (Figure 1c). It is anticipated that images generated from this protocol could be used to measure proximity of proteins to the endoplasmic reticulum, distribution of protein in/on the endoplasmic reticulum and identify protein endoplasmic reticulum interactions. The ability to observe a whole endoplasmic reticulum structure in relation to cellular proteins will provide a wealth of information about protein location, interaction and function in relation to this extensive and essential organelle.

Acknowledgements

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Figures

Figure 1

ReZolve-ER staining with antibody staining Micrograph showing PNT2 cells stained with ReZolve-ER (A, green in C) and anti-Cytochrome C for mitochondrial detection (B, purple in C).