Troubleshooting Forum

Molecular Biology Techniques Q&A

GFP Imaging in Fixed Cells

This month's question from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) comes from the "Microscopy and Imaging Techniques" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

What fixative will allow visualization of GFP fluorescence? (Thread 13765)

Q I transfected endothelial cells with a GFP-tagged protein, which I expected to be expressed on the cell surface. I selected the cells using specific antibiotics and then fixed with methanol. I then stained the cells with DAPI to ensure that there were cells on my coverslips. However, when I looked at my coverslips through the microscope, there was no green fluorescence at all.

Others have used this vector before and successfully imaged the fluorescence, so I suspect fixation might be the problem. Can someone offer some suggestions for a fixing agent I can use that will allow me to see my GFP-tagged proteins?

A GFP fluorescence is eliminated when the proteins are exposed to acetone and GFP is very sensitive to dehydration as well. I suggest that you fix the cells in formaldehyde. I have used formalin and glutaraldehyde successfully in the past. Be certain that your cells don't get dehydrated since that will entirely eliminate the fluorescent signal.

A In the past, I have used this fixative solution and it worked well. I did not design it for GFP, but I had it on hand and tried it. Since it worked, I continued using it.

2.7 ml 37% formaldehyde
0.4 ml 25% glutaraldehyde
46.9 ml PBS

Incubate the cells with fixative for 5 min at 4 degrees, rinse with PBS, and store the cells in PBS. This should enable you to see native GFP fluorescence. Of course, you can use any fixative you want if you are going to do immunocytochemistry with an anti-GFP antibody.

A Have you tried paraformaldehyde? It preserved my GFP tagged protein quite well.

A Is your vector stably or transiently transfected? It should express GFP unless there is a problem with the vector. Did you check the living cells for a signal before you fixed them? You should look at them if possible to make sure they are expressing the protein as expected. It is important to determine whether the problem is with the fixative or the initial transfection or expression. If you don't see fluorescence in the unfixed cells, you should sequence the vector to look for potential problems.

A I fixed my cells in methanol for 30 minutes at room temperature and can still see my GFP-fusion protein in 293 transfected cells 6 months later.

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A Contrary to the previous post, I find that methanol quenches GFP. Why don't you just use an antibody against GFP? Then you can continue with methanol fixation. Otherwise, you might try 4% paraformaldehyde.

A What form of GFP are you expressing? I previously worked with one of the original bacterial codon optimized GFPs. When I fixed it with methanol, I also lost the GFP signal completely from my transformed bacteria. But when I changed to EGFP, I no longer had a problem the signal following methanol fixation.

With the older GFPs, methanol not only fixes the tissue, but I think it denatures the protein simultaneously. Clearly, a denatured fluorophore can no longer fluoresce.

Try fixing with 2% formaldehyde in PBS for 10 minutes at room temperature. This works for me when using the older GFPs and I think it should probably work for you.

Q When you fixed your GFP expressing cells with glutaraldehyde, did you encounter problems with autofluorescence when you viewed them under a confocal microscope? I tried this method and had significant autofluorescence interfering with imaging. Is it possible to quench the autofluorescence? With formaldehyde, I read that I could simply quench the autofluorescence with ammonium chloride treatment, but I couldn't find a quenching reagent for glutaraldehyde.

A I routinely use 50mM ammonium chloride in one of the washing buffers after formaldehyde and glutaraldehyde fixation for quenching the glutaraldehyde autofluorescence and also for deactivating or blocking unreacted aldehyde groups in the tissue. If you want to read the details, you may check Roth et al. Enhancement of Structural Preservation and Immunocytochemical Staining in Low Temperature Embedded Pancreatic Tissue. *J. Histochem. Cytochem.* 29,663-671, 1981.

Another interesting reference is Tagliaferro et al. Immunofluorescence and glutaraldehyde fixation. A new procedure based on the Schiff-quenching method *Journal of Neuroscience Methods*, 77:2, 1 December 1997 (191-197).

A It is well documented that GFP is quenched by methanol in some people's hands. This was my experience as well. Paraformaldehyde usually works well, but be aware that liquid formaldehyde can contain up to 15% methanol, which will also damage your fluorescent signal.

A *J. Histochem. Cytochem.*, 51: 271-274 (2003) describes a procedure for labeling green fluorescent protein expressing cells in 1-micron thick zebrafish sections. The authors were able to detect fluorescence and later observe the sections by electron microscopy using a 10 nm gold-labeled secondary antibody. In that case, GFP fluorescence survived fixation in 4% paraformaldehyde with 0.1% glutaraldehyde and could be visualized directly by fluorescence microscopy.

A I work with worms expressing GFP markers. The GFP signal does get lost during fixation, especially when using methanol, but some loss of signal is observed with many fixatives. To work around this, after fixation, I incubate the worms with a FITC conjugated anti-GFP antibody from Abcam at 1/1000 dilution

for at least one hour followed by DAPI. Invitrogen also offers a good anti-GFP antibody conjugated to AlexaFluor.

A Several of the suggestions offered in the previous posts should work. I would like to add that fixing in 1% paraformaldehyde in PBS for 20 minutes works well in my hands. If you plan to follow up with a phycoerythrin- or rhodamine-labeled antibody stain against another protein, you should permeabilize the cells with 0.25% TritonX100 in PBS. This will help the antibodies and DAPI counterstain enter the cells and give you a better chance at a successful stain. If you still have quenching problems with either GFP or the fluorescent-labeled secondary antibody, Vectorlabs make a good anti-quenching mounting medium that you can use for coverslipping. I would also recommend avoiding phycoerythrin-labeled antibodies since that fluorophore quenches very easily under intense light.

A I have had very good luck with 4% paraformaldehyde when made fresh from powder. I dissolve it in Hank's BSS at 65°C, then cool it to room temperature. All of my constructs include EGFP. I choose cells with visible EGFP fluorescence and rinse them with Hank's BSS, fix them for 15 minutes at room temperature, and then rinse them several times with Hank's BSS. Finally, I rinse in PBS before mounting the cells or examining them by microscopy. For immediate imaging, you can seal your coverslips with PBS, but I found that Fluoromount is easy to use and keeps the GFP bright for at least a week when stored at room temperature. This is useful if your confocal microscope is located away from lab or is heavily scheduled. It also allows you to check the coverslips again later since EGFP, DAPI, and MitoTracker Red remain visible.

A Why do you want to fix cells for detection of GFP? There really is no need for fixing them; just image the live cells. If you want a nuclear stain in addition to the GFP signal, you can use Draq5, which is cell permeable and will give you the same information as DAPI. The only reason for fixing cells to detect GFP is if you also need to stain intracellular antigens.

You mentioned that your GFP-tagged protein is expressed on the cell surface of endothelial cells. The cells are adherent, correct? Did you trypsinize your cells to detach them? If so, almost all of your cell surface proteins would be digested by the trypsin and that might be the reason for the loss of signal.

A Methanol is not a fixative. Instead, it immobilizes proteins and denatures them. Since GFP is a protein, it will not fluoresce when it gets denatured. You should not use methanol or any other denaturing agent with GFP. Instead try 4% paraformaldehyde, electron microscopy grade. You must prepare it fresh for each use, using an osmotically compatible buffer at the proper pH. You may also include a low concentration of glutaraldehyde if desired.

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