FOCUSCLEAR™ – FAQ

1. **Can I use MountClear™ alone to clear and mount my specimen at the same time?**
   No. MountClear™ is a mounting solution specially designed for mounting specimens cleared by FocusClear™. If a specimen is cleared directly with MountClear™, the solution will clot very soon and there will not be enough time to reach satisfied transparency results.

2. **Can I use MountClear™ to replace the standard glycerol-pPDA as the mounting solution?**
   Yes. For best result, clear your specimen in appropriate amount of FocusClear™, then transfer it into MountClear™.

3. **What is the refractive index of Immersion Solution-M?**
   The refractive index of Immersion Solution-M is 1.43.

4. **I found MountClear™ with very unusual consistency, somewhat like jelly. At room temperature, MountClear™ clouds up and FocusClear™ crystallizes in the bottle. Is this normal?**
   Cloudiness of FocusClear™ and MountClear™ are normal when stored in low temperature. Storing at room temperature over a long period of time may result in a bit of cloudiness too. They can be completely dissolved in a hot water bath at 55 °C for 30 min without affecting their effectiveness.

5. **I have experienced a great deal of difficulty in trying to get rid of autofluorescence in Drosophila's whole brains. What should I do?**
   Autofluorescence comes from several different possibilities. First, glutaraldehyde fixation will induce autofluorescence of most biological tissues. Second, eye pigments from red eye flies show strong autofluorescence. In this case, use white eye flies or remove the eye pigments. Third, if you use nail polisher to seal the cover slip, some brands may contain fluorescence compound that will contaminate your sample. Simply try a different brand.

6. **As I anticipated, I did not see the level of tissue clearing as you described. The brains became clearer, but they did not become transparent.**
   To obtain complete transparency, the brain needs to be well fixed and penetrated. A recommended protocol was described in the *Illustration* (please contact technical services for this information). If the specimens are fly brains, a simple procedure is also available: A short pre-fixation in 4% paraformadehy followed by another fixation in 4% paraformadehyde

with 0.25% Triton-X100 overnight offers the best result. Two-hour fixation offers reasonably good clearing effect with FocusClear™.

7. **Could pre-fixing or post-fixing pose any additional problems with FocusClear™?**
   In some cases like samples for immunohistochemistry, a short fixation is required for the following immunolabeling treatment. In that case, you should then do a post-fixation at the end of immunohistochemistry before the FocusClear™ treatment. Fixation before or after dissection or even after immunohistochemical staining should not make much difference for the effect of FocusClear™. Most importantly, your sample should be well fixed before clearing.

8. **Will FocusClear™ work on live plant tissue?**
   In general, FocusClear™ is suitable for fixed samples. However, we have few experiences to treat FocusClear™ on living plant tissues, and the short-term treatment did not cause acute killing.

9. **Does FocusClear™ work with Drosophilae embryos?**
   Definitely.

10. **I wonder whether you have tried Alkaline Phosphatase.**
    We did not try any experiment with alkaline phosphatase. However, FocusClear™ can easily clear the biological specimens and help to visualize the internal fluorescence and chromophore.

11. **How should I seal the edges of the cover slip and will not run the risk of affecting the mounting medium in the chamber?**
    For the nail polish sealing, slightly press the cover slip. The redundant FocusClear™ or MountClear™ will be pressed out, and the cover slip will be tightly in touch with the spacer. It means there is no room to form channels to leak nail polish into inner chamber while sealing. A hint for using nail polish is to seal three times with very thin layer. That will result in drying rapidly and less contamination. In fact, MountClear™ can be used without nail polish sealing. While the samples are embedded in MountClear™, the mixtures already clot (low temperature will facilitate the clotting).

12. **What is the best way to clean FocusClear™ and MountClear™ if some of them have contaminated the surface of the cover slip?**
    Contaminations of FocusClear™ and MountClear™ can be easily cleaned with water.

13. **I just had my first look at a FocusClear™ treated brain on the multi-photon microscope. There was no DAPI staining. Does FocusClear™ interact with DAPI? I used DAPI and PI as these are good fluorochromes to stain nuclei.**
    Since the binding between DAPI and DNA is weak and often dissociate during long-term

storage, the post-fixation is needed (4 % paraformaldehyde). You can also shorten the FocusClear™ treatment time. In general, a cockroach brain with a thickness of 500 micrometer can be cleared within 1~2 hours. If the specimen is thin like fly brains, the clearing time is about 10 minutes.

14. **After applying FocusClear™ to my specimen, I found that bleaching was a lot faster than my control. Why did this happen? And how should I avoid it?**
Because of more efficient excitation one would expect faster bleaching. The solution of this is to use lower laser power. For example, use 488 laser equipped in Zeiss LSM 510 confocal microscopes at 10-15% attenuation of 75% laser power. This would prevent most bleeching problem. Since the fluorescence signal is much stronger in FocusClear™, even with low excitation laser, the image quality as well as resolution is greatly improved comparing with samples in glycerol.

15. **Why was there increased background fluorescence on my sample when I used FocusClear™?**
Since FocusClear™ treated samples will become completely transparent, it is expected to see more fluorescence from focus as well as out-of-focus planes. This becomes a problem when using a conventional fluorescence microscope because it often becomes too bright. The increased background fluorescence is coming mostly from out-of-focus planes. In fact, in some cases, fluorescence signals become so strong that we cannot even look at the samples under a conventional fluorescence microscope directly without using a ND filter. Thus, for the observation of thick samples under conventional fluorescence microscope, FocusClear™ is only useful for increasing detection sensitivity from samples with weak signals, which is often the case for many samples. On the other hand, strong fluorescence signals become a great advantage when using a confocal microscope that allows the use of a smaller pinhole size to filter out all the out-of-focus signals. Under this situation, in fact, many of our users found that the background fluorescence of the focus plane of samples mounted in FocusClear™ is much lower than the samples mounted in glycerol.

16. **Why were the phase and DIC images severely affected as I anticipated?**
This is expected if samples cleared with FocusClear™ are directly observed with DIC optics. It becomes a great advantage when samples are labeled with non-fluorescence chromogens such as lac Z, DAB, and NBT before clearing. In our hand, DIC imaging is routinely used for observation of chromogenic-labeled structures in thick samples such as a whole-mount Drosophila's brain showing lac Z stained gene expression patterns. Again, these DIC Images are superior when samples are mounted in FocusClear™ than mounted in glycerol.