Light Microscopy: Tips

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Recommended coverslip thickness:

Most microscope lenses have the designation 0.17 printed somewhere on the lens. This number indicates the expected glass coverslip thickness (mm) that was used to calculate the optical corrections in the lens. Using coverslips of the wrong thickness, particularly with lenses that have numerical apertures higher than 0.65, can add optical aberrations to your images. For example, at a numerical aperture of 0.95 (typically a 40X dry lens), a 0.01 mm difference in thickness reduces image formation by 45% from the ideal image[1].

The Fisher Scientific web site shows that the thicknesses of their glass coverslips are: **#1** (0.13-0.17mm), **#1.5** (0.16-0.19mm) and **#2** (0.17-0.25mm). Other vendors have similar numbers *(the ranges are one standard deviation).*

We recommend that labs always use #1.5 thickness glass coverslips. This thickness is particularly important with cells cultured on a coverslip, since they are attached to the surface of the coverslip and are very close to the microscope objective. Note, there are actually some individuals who use a micrometer to ensure that their coverslips are the correct thickness.

If you need to use plastic coverslips for cells that do not grow well on glass: Instead of mounting the coverslip in the traditional way, with the cells down (against the slide), mount the coverslip with the cells facing up. Then place the mounting media and a glass coverslip over the top. This solution works very well for confocal microscopy, but transmitted light techniques such as DIC will suffer a bit. This is because DIC uses polarization and plastic is a polarizing substance. Also, if you are doing widefield fluorescence work and you have plastic coverslips that are autofluorescent, you will still have a problem with the autofluorescence, but it won't be quite as bad. *(This suggestion came from the Confocal Listserver, Oct 5, 2004)*

1) G.S. Benham, pg 257 in Cell Biological Applications of Confocal Microscopy, Edited by B. Matsumoto, Academic Press, 2002.

Coverslip mounting media:

There are a number of different types of mounting media.

- Some types of media harden to hold the coverslip firmly in place
- Some types of media include an antifade reagent to help immunofluorescence last longer
- Some types of media use different solvents *(water, glycerine, organic chemicals like xylene)* because the stains in the sample preparation are sensitive to a particular type of solvent. These types of mounting media have different refractive indices. Mismatched refractive indices can cause problems with image formation in the microscope.

Because there are so many types of mounting media, it is important that the media be matched to the intended use. Since most of our users are interested in mounting their own immunofluorescence (IF) or immunohistochemistry (IHC) preparations, the following information will be directed toward that group.

Users that are mounting IF or IHC preparations will usually want to avoid organic solvent-based mounting media and instead use media that is water, glycerine or poly-vinyl alcohol based *(e.g., Mowiol).* An important consideration is the refractive index (RI) of the mounting media. The RI of glass is typically 1.515 and oil-immersion lenses require oil that is of a similar RI. The refractive index of air is 1.0, water is 1.34, and glycerine is 1.41. The RI mismatch between the glass and the mounting media will cause significant optical aberrations with a high numerical aperture oil immersion lens, something to consider if confocal or deconvolution microscopy is planned. The aberrations increase as the microscope images deep into the sample. Use of an expensive water or glycerine immersion lens with a correction collar can compensate for this problem.

The best way to avoid RI problems is to mount your samples on the coverslip, rather than on the slide. Unfortunately, it is not always feasible to mount samples this way. The next best thing would be to use the smallest amount of mounting media that will fill the space between slide and coverslip. This amount may be determined empirically by experimentation with blank slides and coverslips, allowing for shrinkage if the mounting media is a type that hardens.

The following anecdotal observations have been reported about commonly used mounting media for IHC or IF:

- Use of fingernail polish to seal around the edges of coverslips has been reported to quench GFP fluorescence *(Cytometry. 35: 353-62).* Others have not seen this problem *(Confocal Listserver).*
- Quantum Dots should not be mounted with VectaShield or ProLong, as these quench the fluorescence of the Qdots. The company recommends Mowiol[2] or the Sigma-Aldrich PVA mounting media (w/ DABCO). *(telephone communication)*
- The antifade additive p-phenylenediamine is not usable for Cyanine dyes *(CY3, CY5, etc)*. This is the main ingredient in Vectashield.[3]
- Crystal Mount is not good for several red dyes (PE, PC, APC) or fluorescent proteins.[3]
- Prolong is good for Alexa dyes, but bad for fluorescent proteins.[3]
- VectaShield is reported to not work well with Texas Red *(Confocal listserver).*
- The refractive index of home-made Mowiol mounting media can be highly variable *(per Tom Donnelly of Applied Precision, Confocal Listserver).* Mr. Donnelly is also skeptical of the listed refractive index values for any of the PVA mounting media *(personal communication).*
- Users are encouraged to review the product insert, or contact the vendor of your fluorescent dye(s) to see which mounting media they recommend.

The following recommendations are for placing the coverslip onto the microscope slide:[4]

- Inverted microscopes require you to place the slide on the microscope stage with the coverslip down. When assembling your slides, do not place the coverslip all the way to the end of the slide. Leave 3-5 mm of the slide free from a coverslip at each end. This allows the slide to lay completely flat and stable on the microscope stage.
- There are a number of ways to do mount coverslips on microscope slides and the consensus seems to be that whatever way that works for you is fine, as long as it doesn't create bubbles in the mounting media. Bubbles are bad because the refractive index of air is a very poor match and small spherical bubbles can act like tiny lenses that scatter light. Tom Donnelly (*Applied Precision*) says that the "…trick I use is to put a very small amount of mountant *(10ul at the most)* on the coverslip and bring the slide down to the coverslip and let the surface tension pull the coverslip up. This works especially well if you are using aqueous media."
- Use just enough mounting medium to fill the space under the coverslip, but not so much that the sample moves around.
- Mount the coverslip as close as possible to the sample surface. An extra 20µm of thickness, the result of adding 10 µl too much mounting medium, can make the sample impossible to image at 100x. *(This is due to the very short working distance of high magnification oil-immersion objective lenses.)*
- The other extreme is to be so "stingy" with the mounting media that you create air bubbles at the edges of the coverslip. With too little media you may be tempted to press down on the coverslip to ensure a tight seal. This pressure can crush or distort the three-dimension structures in the sample.
- Seal the coverslip to the slide with clear nail polish, or other hard sealant. Some labs working with solvent sensitive protocols use a sealant called VALAP[5], made of equal parts of paraffin, vaseline and lanolin. VALAP has the advantage of being very easy to clean off of microscope objectives, whereas nail polish is notoriously difficult to remove.
- Once the mounting media and sealants have completely dried, carefully clean left over solvents/mounting media/fingerprints off the coverslip and/or microscope slide. This can be done by using a lint-free wiper *(e.g., Kimwipe)* lightly wetted with microscope lens cleaner, ethanol or commercial window cleaner (e.g., Windex).

2) A Mowiol recipe: http://cshprotocols.cshlp.org/content/2006/1/pdb.rec10255.full?text_only=true
3) Modified from: Oregon Health Sciences University (web page no longer exists)
4) Modified from: http://cpmcnet.columbia.edu/dept/gsas/anatomy/Files/results.htm#mounting
5) VALAP: https://nic.med.harvard.edu/VALAP

Don't mix immersion oils from different vendors:

Be careful when reviewing your samples at high magnification on two different brands of microscope. If you find that you need immersion oil to see fine details in your sample, please do not "mix" two different brands of immersion oil. Vendors tell us that they have seen instances where the two different oils combine on the lens surface and form a sticky substance that's very hard to clean off the lens. If you find you need to use two different microscopes, try to first clean the original oil off the slide using lens cleaner, followed by a wash with a gentle stream of 100% ethanol.

Mixing and matching microscope objectives:

We are occasionally asked the question "can I use an objective lens from 'company X' on a 'company Y' microscope?" The answer is "sometimes" and "it depends". Just because the threads on the objective allow it to be screwed into the microscope nosepiece does not mean that the lens will work as hoped. Images viewed with mixed equipment may be acceptable for casual observation "by eye", however, use of digital or film photography will most likely reveal a number of optical aberrations. Here are a few issues to be aware of:

Microscopes purchased in the last 10-15 years are probably "infinity corrected". Older microscopes are "fixed tube length". Infinity corrected lenses will have the ∞ symbol on the barrel of the lens. Fixed tube length lenses will usually have the number 160 *(occasionally 170 or 180)* on the lens barrel. *(For an interesting essay on identifying the properties of objectives, see: http://micro.magnet.fsu.edu/primer/anatomy/specifications.html)* **LENSES MUST BE OF THE SAME TUBE LENGTH AS THE MICROSCOPE.**

The major microscope manufacturers use different optics in the microscope to correct for chromatic aberrations *(see: http://micro.magnet.fsu.edu/primer/anatomy/aberrations.html).* As a consequence, users need to know which manufacturer's lenses are corrected in a similar fashion to avoid adding optical aberrations to their microscope. The following table summarizes objective lenses that can **probably** be interchanged between microscopes with few optical problems.

Type	Compatible lenses	Comments
160mm fixed tube length	Leitz, Olympus, Zeiss	Olympus & Zeiss are considered "closest" in compatibility. Note, older American Optical (AO) microscopes used infinity correction, but this may not be marked on the lens.
Infinity corrected	Nikon CF	Objective thread does not match that of other manufacturers. Adapters for the objective thread are available, however, non-Nikon lenses may not be fully optically compatible.
Infinity corrected	Leica, Olympus, Zeiss	Olympus & Zeiss are considered "closest" in compatibility.

Note: the information in this table is based on the empirical experiences of scientists and vendors in the field, as well as some known general characteristics of the design of vendor's microscopes. We are not aware of a definitive source for this type of information. Users should be aware that the best optical characteristics of an objective lens will almost always come when it is used on the microscope system that it was designed for. Many thanks to **Dave Chiasson** (formerly of Carl Zeiss Inc), **Dr. Russell Carey** of Scientific Instruments (formerly with Olympus America), **Kate Hendricks** of Nikon USA, and **Dr. Bob Chiovetti,** of SW Precision Instruments (http://www.swpinet.com/) for their assistance with this table.

Remember that the objective lenses are a critical component of the image forming optics of a microscope. The lenses should be handled and cleaned with great care *(see below).*

Cleaning Microscope Objectives:

Poor image formation in a microscope can often be attributed to the objective lens having dust, fingerprints, or being contaminated with mounting media or immersion oil *(non-oil objectives)*. Microscopes should be thoroughly cleaned by a service professional about once a year, usually for a cost of a little over $100. While the service person is cleaning the microscope, ask them for a hands-on tutorial in how to clean the eyepieces and lenses of the microscope.

We have routinely cleaned microscope lenses using Sparkle™ cleaner (http://www.glasscleaner.com/), which is available at ACE® Hardware, Sears, and True Value® hardware stores. For lenses that have oil on them we have used absolute ethanol. If these two methods do not work to clean the lens, we defer to the manufacturer's certified service professionals. **Use of stronger organic solvents on microscope lenses can loosen the glue used to hold the lens elements in place and ruin the lens!**

The following websites provide information about cleaning microscope lenses. They are provided "as is" with the warning that lenses are easily damaged. Some of these sites advocate more aggressive cleaning methods than we are comfortable with; however, there are many schools of thought about the best way to clean microscope lenses. **Please be VERY careful cleaning the optical surfaces on your microscope!** Be aware that the higher magnification lenses on research-grade microscopes can cost thousands of dollars.

- http://www.microscopy-uk.org.uk/mag/artfeb04/cdclean.html
- http://micro.magnet.fsu.edu/primer/techniques/fluorescence/troubleshoot.html
- http://micro.magnet.fsu.edu/primer/anatomy/cleaning.html

For a Southern Arizona list of microscope vendor & service contacts see:
http://swehsc.pharmacy.arizona.edu/swehscfiles/docs/ci/az_lm_vendors.pdf

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Resources:

Molecular Expressions *(sponsored by Florida State University, along with Leica, Nikon, Olympus & Zeiss)* is the premier light microscopy tutorial site on the WWW. See their website at: http://micro.magnet.fsu.edu/primer/index.html

Optimizing Light Microscopy for Biological and Clinical Laboratories *(May 1997)*, by Barbara Foster, American Society for Clinical Laboratory Science, Kendall/Hunt Publishing Company; ISBN: 0787235385.

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About the author:

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