Multi-Immersion Objectives

Applied Scientific Instrumentation (ASI) worked with Special Optics to develop two immersion/dipping objective lenses for light sheet microscopy. They were intended for use with cleared tissue samples, but are useful for live cell imaging when a very long working distance or RI matching is important. These objectives work in any refractive index media without a correction collar.[1)] The first objective (54-10-12) has nominal NA 0.4 and has been offered since October 2017 at the price of \$15k. The second design (54-12-8) has nominal NA 0.7 and became available June 2019 for \$24k. ASI is the sole distributor of these objectives but will sell them freely to anyone interested, including home builders and companies.

The original goal was isotropic ~1 micron resolution at least 5 mm deep into slabs of cleared tissue. Doing this with a dSPIM/diSPIM geometry (two orthogonal identical objectives each 45 degrees above the sample) requires a modest NA objective with extremely long working distance and tapered shape. We designed the objective lens accommodate a wide variety of imaging media, which is an important feature given the variety and rapid development of clearing protocols. The first objective lens has been very well received but some users have expressed interest in higher resolution, which led to the design of the second objective lens with higher NA at the expense of some working distance and field of view. These are unique objectives because of the combination of multi-immersion capability, very long working distance, and mechanical profile amenable to light sheet imaging. [2)]

54-10-12 Specifications

Specification	Value	Comments
Numerical Aperture	0.4 @ RI 1.45	0.37 – 0.43 over RI range
Immersion Media RI	1.33 – 1.56	will also work in air or any media RI
Chemical Resistance	very high	Aqueous and organic solvents including DBE and more (see list)
Effective Focal Length	12 mm @ RI 1.45	15.3x – 17.9x over RI range w/ 200 mm TL
Working Distance	12 mm (for all RI)	> 5 mm imaging depth with flat sample, 12 mm Ø sphere
Field of View	1.2 mm Ø	
Spherical Correction	480 – 1000 nm	Diffraction-limited for most media and λ
Chromatic Correction	480 – 720 nm	Performance varies by media, optimized for CLARITY and TDE
Correction Collar	None	Designed for dipping (immersion w/o coverslip)

Specification	Value	Comments
Form Factor	Nikon style	61.6 mm parfocal distance, M25 x 0.75 threads, 38.5 mm OD, ~225 g

The mechanical drawing and corresponding 3D CAD file are available. [3]. Of interest is a drawing of how two of the 54-10-12 objectives co-focus which is posted on dispim.org. Zemax black box files are available from ASI upon request.

54-12-8 Specifications

Specification	Value	Comments
Numerical Aperture	0.7 @ RI 1.45	0.64 – 0.75 over RI range
Immersion Media RI	1.33 – 1.56	will also work in air or any media RI
Chemical Resistance	very high	Aqueous and organic solvents including DBE and more (see list)
Effective Focal Length	8.4 mm @ RI 1.45	22x – 26x over RI range w/ 200 mm TL
Working Distance	10 mm (for all RI)	2 mm imaging depth with flat sample, 10 mm Ø sphere
Field of View	1.0 mm Ø	
Spherical Correction	480 – 1300 nm	Diffraction-limited for most media and λ
Chromatic Correction	480 – 720 nm	Performance varies by media, optimized for CLARITY and TDE
Correction Collar	None	Designed for dipping (immersion w/o coverslip)
Form Factor	Nikon style	83 mm parfocal distance, M25 x 0.75 threads, 38.5 mm OD, ~383 g

The mechanical drawing and corresponding 3D CAD file are available. Of interest is a drawing of how two of the 54-12-8 objectives co-focus which is posted on dispim.org. Zemax black box files are available from ASI upon request.

Magnification

Both objectives are infinity-corrected and designed to be used with an infinity-corrected tube lens as is common practice in modern microscopes. The magnification is given by the ratio of the effective focal lengths of the tube lens and that of the objective lens. We assume the Nikon convention of 200 mm focal length tube lens, which ASI uses by default on SPIM systems. However, if a different magnification is needed then a different tube lens can be used. ASI offers a variety of suitable tube lenses as do others.

The effective focal length of the two objectives (EFL_{obj}) depends on the medium refractive index (RI) according to the following formula:

54-10-12: EFL_{obj} = 17.4 mm / RI

54-12-8: EFL_{obj} = 12.19 mm / RI

The nominal RI is 1.45 (FocusClear/CLARITY), which means the focal length is 12.0 mm for the 54-10-12 for nominal magnification of 17x. For the 54-12-8 at RI 1.45, the focal length is 8.4 mm and magnification is 24x.

Because the magnification depends on the imaging medium, magnification ideally would be measured empirically. However, for most situations estimating the magnification based on the presumed RI of the imaging medium is probably sufficient.

The reason that the effective focal length (and hence magnification) depends on the refractive index of the medium can be understood in a few different ways. Perhaps the most simple way to understand is to consider the “back aperture” of the objective.[4)] The NA, the EFL, and the size of the back aperture are related by the following well-known equation $BA_{ø} = 2 * NA * EFL$. The NA of the objective is proportional to the RI of the media; this is essentially definitional. The back aperture has a fixed size. For the equation to hold then the EFL must be inversely proportional to the RI, meaning the magnification is proportional to the RI. An alternative way of understanding is to consider an off-axis ray which passes through the center of the surface between the first objective element and the media. The ray will be refracted off of the glass/media surface with an angle given by Snell's law which says the ratio of sin θ to RI is constant. For relatively small angles, sin θ ≈ θ, which means that the outgoing angle of the ray is is inversely proportional to the medium's RI. Hence the the displacement from center of that ray at the image plane is inversely proportional to the RI, which is equivalent to saying that the magnification is changed proportionally by the medium's RI. Incidentally, the same argument suggests that the magnification varies with RI for **all** objective lenses.

Numerical Aperture and Resolution

The numerical aperture (NA) of the two objectives depends on the medium refractive index (RI) according to the following formula:

54-10-12: NA = 0.276 * RI

54-12-8: NA = 0.483 * RI

The diffraction-limited resolution is a function of the lens NA which depends on the immersion medium as described above. The pre-factors can differ depending on the criteria used to define resolution, but common expressions are as follows for the resolution in the lateral (x,y) and axial (z) directions where λ is the wavelength of light and RI is the refractive index of the mounting medium:

$Res_{x,y} = 0.61 * \lambda / NA$

$Res_{z} = 2 * \lambda * RI / NA^2$

From these expressions it is clear that axial resolution is worse than lateral resolution. For 500 nm light with NA 0.4, the lateral resolution is 0.76 µm and the axial resolution (depth of field) is 9.1 µm (at NA 0.7, the numbers are 0.44 µm and 3.0 µm). The dSPIM/diSPIM geometry provides the opportunity to overcome poor axial resolution by combining two views of the same object from orthogonal directions so that each feature is seen from at least one high-resolution vantage point, all without needing to move the sample. Some home-built imaging systems use the similar concept but rotate the sample to be imaged from different directions.

In light sheet microscopy, commonly only a small fraction of the objective's NA is used for light sheet generation. The baseline axial resolution is the depth of field of the detection objective, but can be improved if the light sheet is thinner than the depth of field. Whether or not this happens depends on the illumination NA and hence imaging FOV. In many cases light sheet provides no true resolution benefit even though out of focus florescence will be reduced (“optical sectioning”) which improves

SNR and image quality. There are ways of creating very thin light sheets to increase axial resolution (e.g. Bessel beams) but they generally have other undesirable properties including extra complexity/cost, large amounts of out of focus light, and/or being extremely sensitive to scattering or sample inhomogeneity.

Working Distance

Both of these objective lenses have been designed with very large working distance. A unique feature is that the working distance is the same for all media.

For most objective lenses, the WD depends on the medium's RI due to refraction at the objective/media surface. Realizing this was going to be a practical issue we specifically designed both objectives to have uniform WD for all RI. The optical implementation of this feature is straightforward: the first element of the objective is concave with radius of curvature centered on the focal plane. Thus to first order there is no refraction at that surface and hence the WD is constant for all RI. For the same reason, there is no intrinsic spherical aberration as the medium RI changes, unlike objective lenses with a flat first surface.

Location of Back Focal Plane

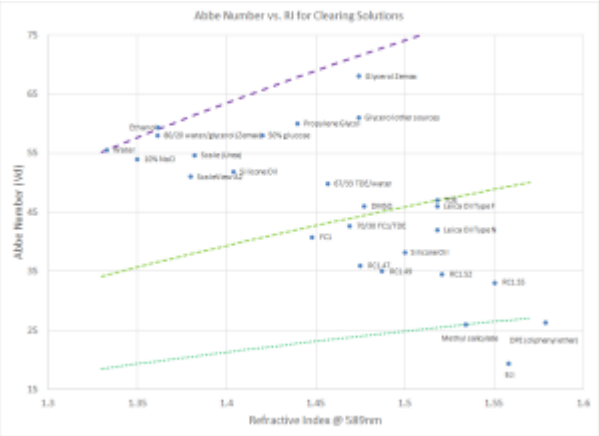
For some applications (e.g. light sheet illumination) it is important to maintain “4f” spacing between optical elements, i.e. space adjacent lenses so that their focal planes line up. If an external lens is positioned with focal position at this plane, parallel rays into the lens pair will emerge from the objective parallel. Although the working distance does not depend on the refractive index of the medium, the location of the back focal plane does depend on it slightly. Per optical simulations for the 54-10-12 objective lens, the location of the back focal plane referenced to flange is approximately 31.07 mm - 38.80 mm * RI + 9.42 mm * RI^2 (negative inside flange). Specifically, this is ~4 mm inside the flange for water, ~5.4 mm inside the flange for FocusClear, and ~6.5 mm inside the flange for ethyl cinnamate (RI 1.56). Per optical simulations for the 54-12-8 objective lens, the location of the back focal plane referenced to flange is approximately -11.75 mm - 22.90 mm * RI + 5.87 mm * RI^2 (negative inside flange). Specifically, this is ~31.8 mm inside the flange for water, ~32.5 mm inside the flange for FocusClear, and ~33.2 mm inside the flange for ethyl cinnamate (RI 1.56).

Spherical and Chromatic Aberrations

Correction collars are commonly used to correct high-NA objectives for spherical aberrations when imaging through a variable-thickness coverslip and/or at different temperatures. Other multi-immersion objectives have a correction collar for different media RI. Even though our objectives have no correction collar, spherical aberrations are still within the diffraction limit for all media and wavelengths simulated due to design features discussed below, most notably the curved first surface. The objectives have minimal chromatic aberration in the NIR spectrum to allow for multi-photon excitation.

Chromatic aberrations are rooted in dispersion, which describes how the exact RI changes with wavelength (commonly reported as the Abbe number). For a specific medium the dispersion can be corrected, but since these objectives are designed to work in many media it cannot be perfectly

corrected for all of them. Chromatic correction could have been improved with a correction collar; this was deemed to add too much complexity for the corresponding benefit. Hence, during the design we could only optimize the chromatic correction for one media, but it turns out that both TDE and CLARITY/Focus Clear are very well corrected for. The dotted line on the plot below shows the approximate “perfect” correction line. Chromatic aberrations can be categorized as lateral color, meaning different wavelengths have slightly different magnification, and axial color, meaning that the focus point is shifted slightly. These chromatic effects scale with the distance from the “perfect” correction line in the plot. The lateral color for water, a rather extreme case as seen from the plot, amounts to ~0.3% change in magnification between 480 nm light and 640nm light for the 54-10-12. We expect that the lateral color can be corrected in post-processing if needed. The axial color remains within the diffraction limit for all media simulated.



Immersion Media

RI Range

A common question is how these objective lenses can accommodate such a wide range of media refractive indexes without a correction collar. There are at least two reasons:

1. They are dipping objective lenses (no coverslip; assumed constant RI from objective to sample). [5)]
2. The first surface is concave with radius of curvature at the focal plane, so to a good approximation no refraction happens at the media/objective surface and so media RI doesn't matter. This point is unique among objective lenses to our knowledge.

Chemical Compatibility

Our objective lenses are designed for direct immersion in index-matching medium. Even though some media used for tissue clearing are corrosive, these objectives can be immersed in most all of them (see list below). The main damage mechanism is media dissolving the glue holding the lens elements in place. This objective was designed to be maximally resistant to media; see list of known safe media below. The only change made between the prototype and production versions of the first (NA 0.4) objective was to improve the sealing materials to allow immersion in more aggressive media.

ASI maintains a list of known safe media (below). Exposing the objectives to the safe media is covered by the two-year warranty on the objectives. **If you want to use a media not on the known safe**

list you can either proceed at your own risk or take the time to arrange for a chemical compatibility test using some special dummy objectives. If the test passes then the media will be added to the known safe list so that others won't have to repeat the test. Contact ASI to arrange a loan of these dummy objectives and instructions for performing the test.

At Society for Neuroscience meetings in 2016 and 2017 there were at least three research groups with preliminary results transferring solvent-cleared tissues to more gentle media for imaging; such protocols could allow e.g. DISCO-cleared samples to be imaged with this cleared tissue objective lens. We expect further developments in this general approach, but we are pleased that tests show that the objective is compatible with DBE, BABB, and other harsh organic solvents.

It is recommended that the objectives be immersed no more than 25 mm into the solution from its tip (37 mm from the focal plane), which is the middle of the surface where the specifications are printed. However, it is thought to be safe to immerse the objectives somewhat more.

Known Safe Media

- water with salt, sugars, and/or other non-aggressive solutes including routine-use biological buffers
- FocusClear (CLARITY)
- glycerol
- CUBIC-1, CUBIC-2, CUBIC-R1, CUBIC-R2
- mineral oil
- silicone oil
- TDE (2,2-thiodiethanol)
- ethyl cinnamate
- benzyl benzoate and BB-PEG (PEGASOS)
- BABB
- DBE (dibenzyl ether)
- other proprietary organic media

1)
but not through a coverslip; this would require a correction collar
2)
Other objectives being used for light sheet imaging of cleared tissue were designed for confocal imaging, with relatively large NA and “fat” form factor.
3)
There is a difference in the profile of the step position in objectives with SN less than 58 (roughly May 2019 transition), the drawing for it is at here. Very early objectives made in 2017 had further slight differences in the nose profile but those were all later retrofitted.
4)
The back aperture or pupil is closely related but not identical to the back focal plane
5)
Most commonly correction collars are to compensate for different coverslip thicknesses because the coverslip has a different RI from the immersion medium