Multiphoton Imaging with Gradient Index (GRIN) Lenses

Application Note

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Introduction

Light scattering fundamentally limits imaging depth in tissue. Since Rayleigh scattering is wavelength dependent, the development of multiphoton imaging, with its use of longer wavelength light, has permitted deeper imaging of living tissue. Two photon imaging routinely images through 1 mm, while three photon imaging permits up to 50% deeper observation. However, scattering still fundamentally limits the depth of these techniques; imaging deeper structures in living tissue, such as subcortical structure, requires a novel approach.

One possible solution is to remove the brain above the region of interest. This method is preferred - by replacing the overlying tissue with homogeneous saline, scattering is minimized, and deep structure is revealed without aberration. However, this technique requires the removal of a significant amount of overlying tissue: one must remove a cone of tissue above the region of interest equal to the numerical aperture of the objective. For experiments that require minimal perturbation of the surrounding brain, a more elegant solution is required.

Gradient index (GRIN) lenses solve this problem nicely. GRIN lenses are so named because of the index of refraction gradient orthogonal to the optical axis (see Figure 1). This index gradient mimics the light shaping properties of a traditional singlet lens: light may be relayed from one focal point to another. GRIN lenses designed to relay light from one end to the other may be used to bypass scattering tissue and acquire images at great depth, without removing as much overlying tissue. Different GRIN lenses that focus light at infinity are critical to the design of miniature implantable microscopes [1]. Combining GRIN lenses with traditional multiphoton imaging, first pioneered independently by two groups lead by Mark Schnitzer and Watt Webb, has allowed unparalleled imaging of deep structures [2, 3].

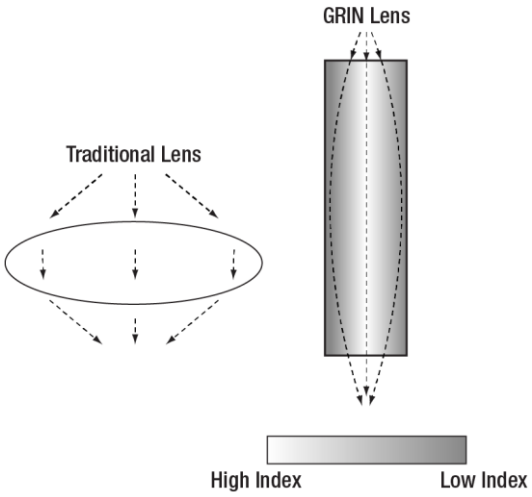


Figure 1. GRIN Lens vs. Singlet. A gradient lens has an index of refraction gradient orthogonal to its optical axis. This gradient bends light inside of the lens. This bending of light can mimic the focusing ability of traditional singlet lenses, where light is bent by the external shape of the lens and the refractive index change between the lens and the surrounding air.

GRIN Lens Specifications

The key design characteristic of a GRIN lens is its pitch. The pitch of a GRIN lens is the fraction of a full sinusoidal wave period of a ray that traverses the lens and determines whether the GRIN lens functions as an objective, or a relay lens (see Figure 2).

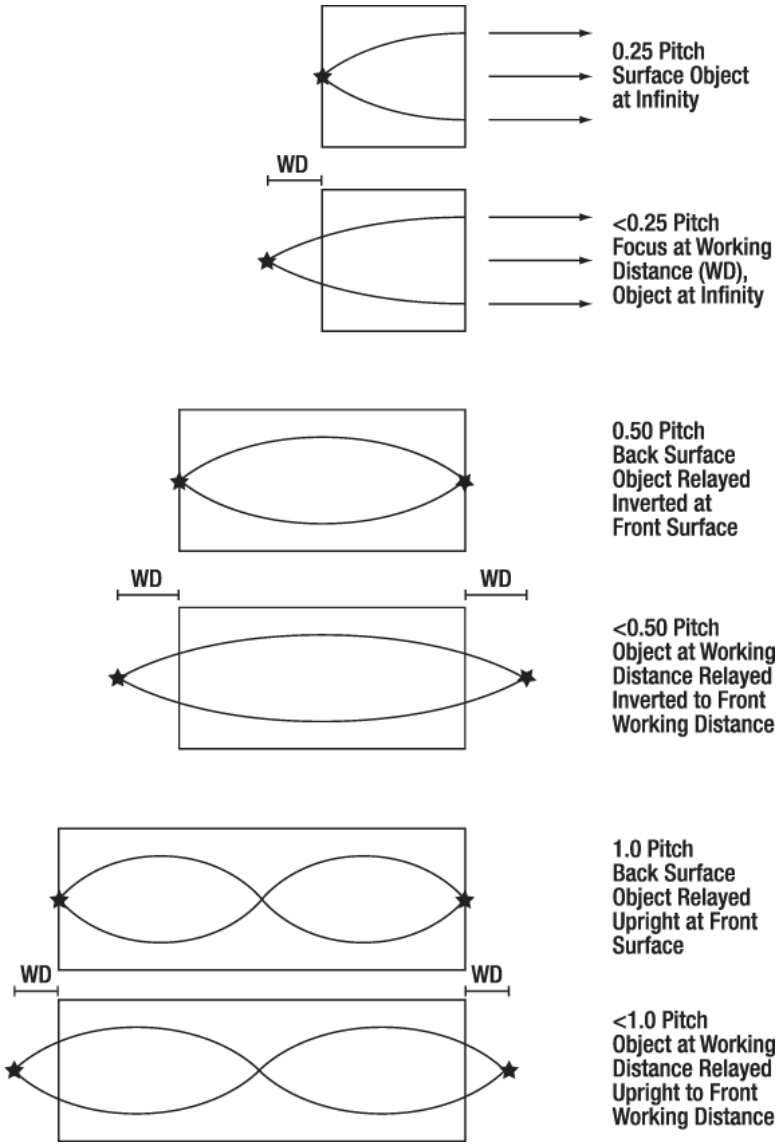


Figure 2. GRIN Lens Properties and Pitch. A GRIN lens' pitch determines its imaging properties. Grin lenses of 0.25 Pitch function like objectives, focusing an object on their front surface at infinity. GRIN lenses with slightly less than 0.25 pitch focus light at a singular working distance to infinity. Grin lenses of 0.5 pitch relay objects from the front surface to an inverted image on the back surface. GRIN lenses of slightly less than 0.5 Pitch relay an object at a singular working distance to an inverted image at working distance on the other side. A GRIN lens of 1.0 pitch relays an object on one end of the GRIN lens to the other end. A GRIN lens of slightly less than 1.0 pitch relays an image at a working distance to the other side.

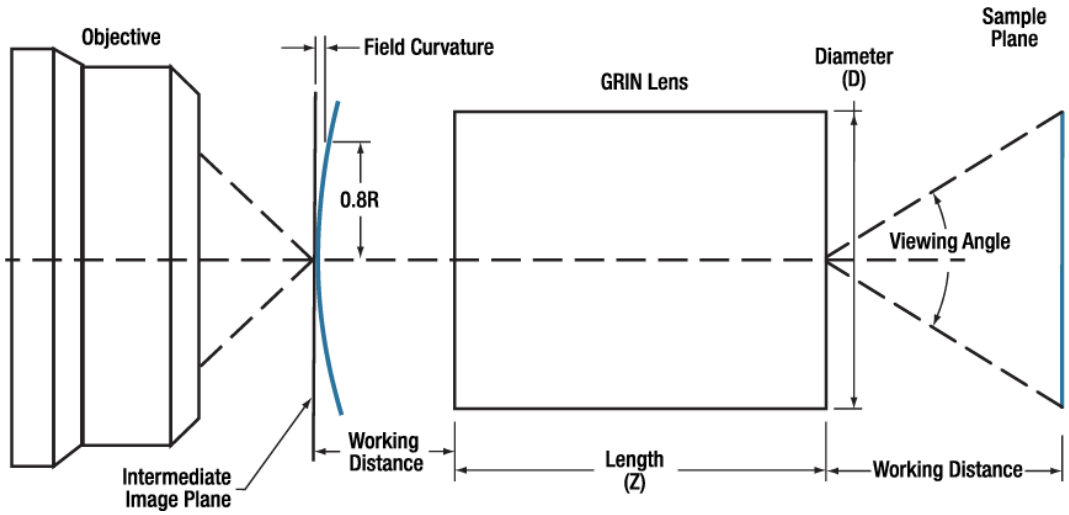


Figure 3. GRIN Lens and Imaging Objective. A GRIN lens aligned with an imaging objective. GRIN lens' imaging properties include working distance, field curvature, and viewing angle.

The pitch of the GRIN lens also determines other critical properties of the GRIN lens, namely its working distance and whether the relay image is inverted. Like objectives, there is only one ideal working distance and focusing at other depths introduces spherical aberrations. In addition, the construction of GRIN lenses limits their field of view (FOV), typically to a radius significantly less than the radius of the GRIN lens itself. Since the GRIN lens field of view changes with distance (see Figure 3), this is often described as an angle rather than a radius. Finally, GRIN lenses typically exhibit significant field curvature. Typically, the GRIN lens is combined with an objective in alignment (see Figure 3), with a traditional microscope objective lens focused at the GRIN lens' intermediate image plane. This arrangement relays the sample plane "seen" at the far end of the GRIN lens to the rest of the microscope.

In this orientation GRIN lenses extend achievable imaging depth well beyond multiphoton and three photon imaging, with relatively modest perturbations of the overlying tissue due to the relatively narrow diameter of the GRIN lens. This permits the imaging of deep structures heretofore out of range (see Figure 4). However, to fully utilize this technology it is critical to understand the aberrations introduced by GRIN lenses and how to mitigate them.

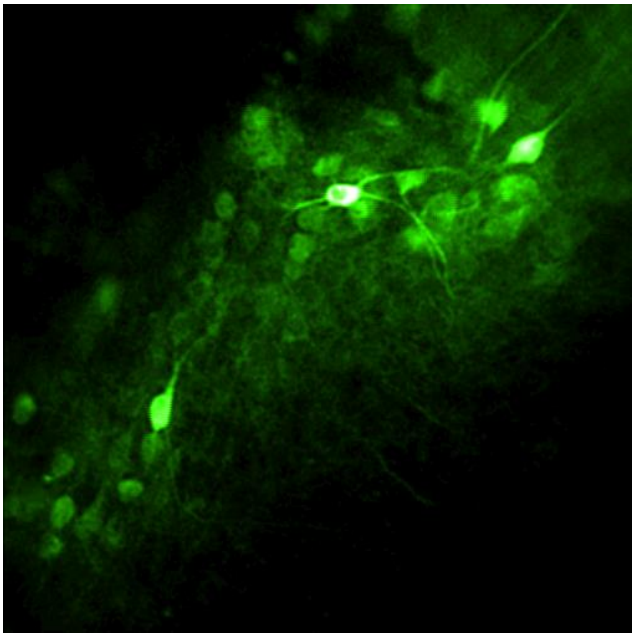


Figure 4. Live Imaging Through a GRIN Lens. Multiphoton imaging of GCaMP-labeled neurons through a 4 mm long, 1 mm wide GRIN lens. Image acquired with Thorlabs Bergamo® microscope equipped with a Thorlabs10X MP objective (TL10X-2P). Image Courtesy of Cody Siciliano, Vanderbilt University.

Implementation

The primary consideration when imaging through a GRIN lens is alignment. Two key elements require alignment when using a GRIN lens. The center of the GRIN lens axis should overlay the tissue of interest, and the objective imaging axis should align with that of the GRIN lens.

Because the optical aberrations of the GRIN lens dwarf that of the objective, it is of vital importance to center the GRIN lens over the target of interest. In practice, this is difficult to optimize, as the GRIN lens is often embedded in a surgically implemented canula, usually cemented in place. Post-surgery, it is impossible to reposition the lens. It is worth noting that the worst aberrations in a GRIN lens arise from off axis aberrations, primarily astigmatism [4]. This astigmatism will decrease both resolution and multiphoton efficiency (brightness). Moving the objective over the region of interest may slightly improve the brightness of the ROI but will not recover the aberrations (see Figure 5).

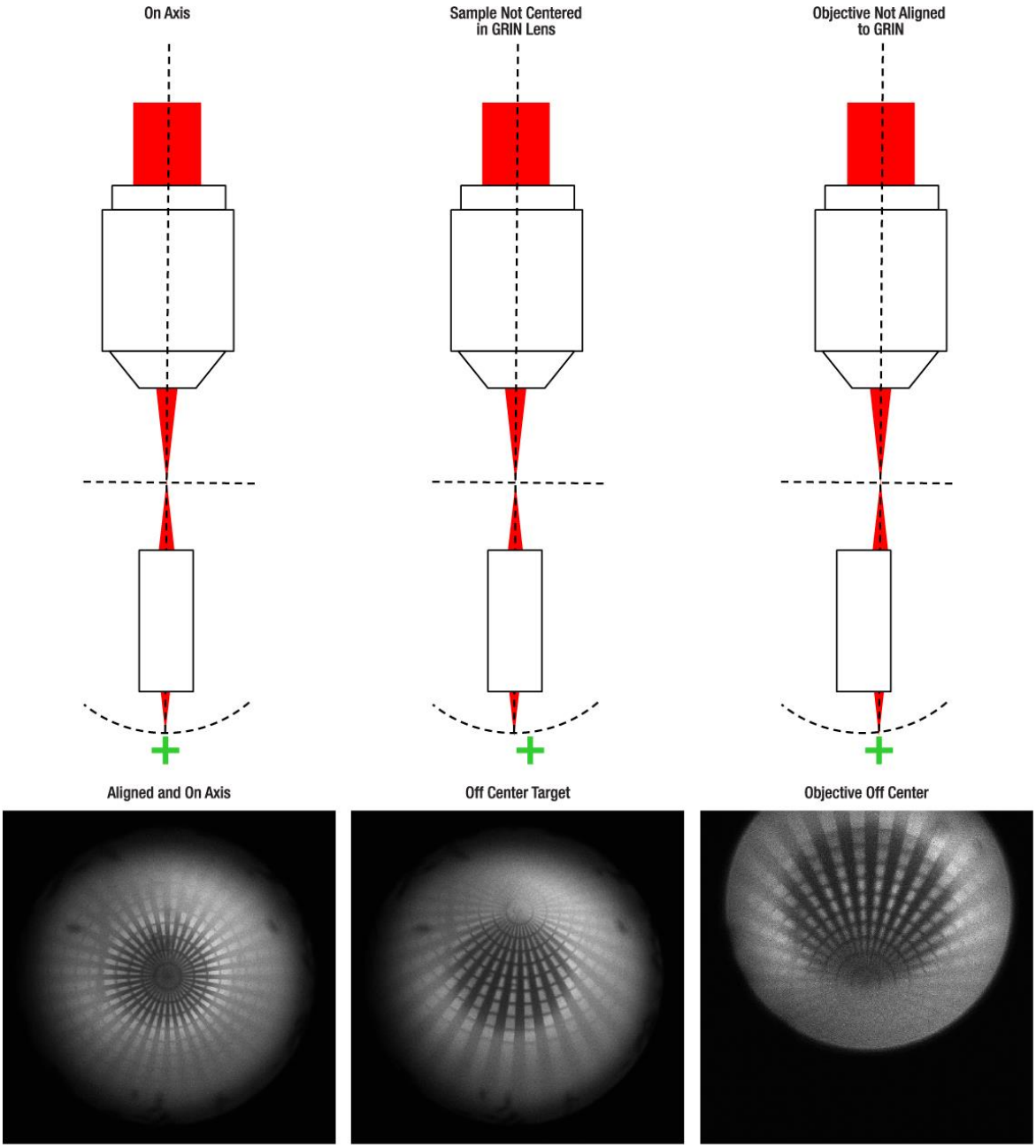
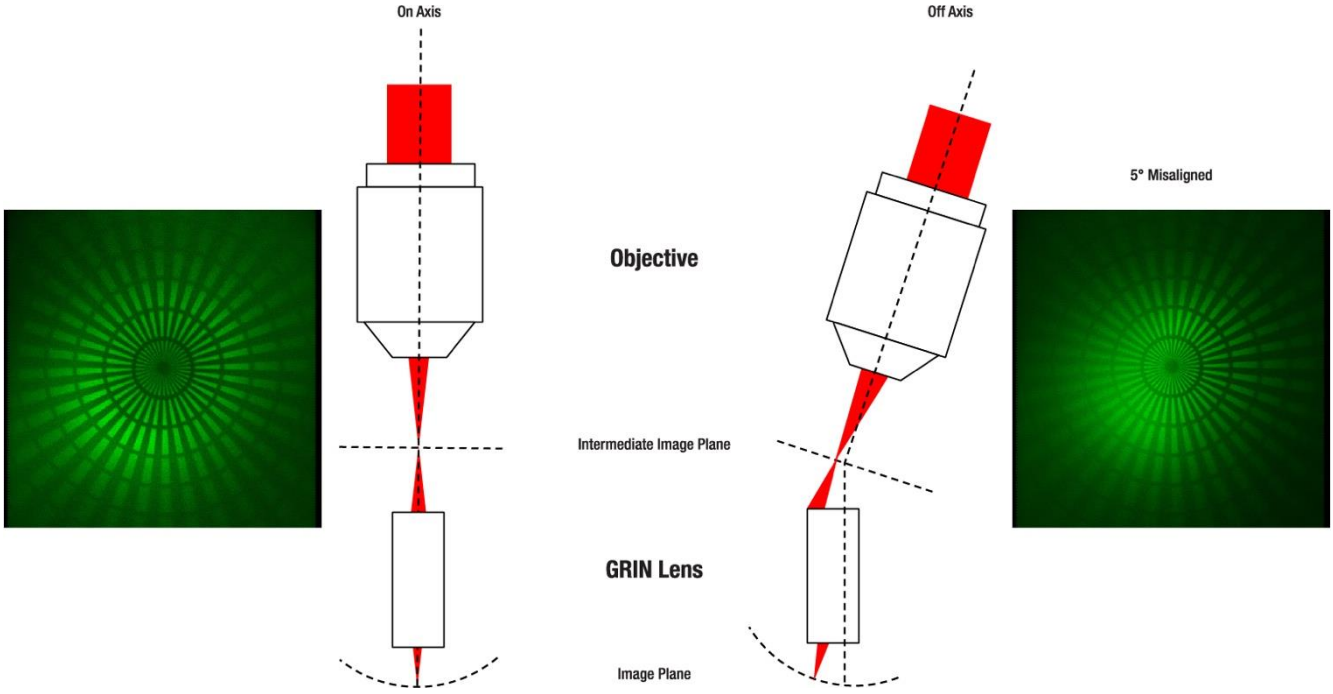


Figure 5. GRIN Lens Imaging Target On and Off Axis. Thorlabs Star Target (R1L1S1P) imaged through fluorescein, a Thorlabs GRIN lens (G1P11), using a Bergamo® multiphoton microscope. The curvature in the imaging field curves the imaging plane away from the target. The resolution is best in the center of the GRIN lens FOV and moving the microscope objective in Y or Z does not recover the resolution lost at the edges of the GRIN FOV.



On Axis GRIN Lens Imaging

Optimal imaging through a GRIN lens also requires aligning the objective with the optical axis of the GRIN lens (see Figure 6). Because the imaging plane of the objective is relayed by the GRIN lens, a misaligned objective will alter the imaging depth across the GRIN lens FOV. This causes multiple problems: First, as one is not imaging into the tissue straight the imaging depth varies across the FOV. Since the tissue is scattering, the shallow side closer to the GRIN lens face receives much more effective power. Second, the effective magnification varies across the field of view; see Figure 6.

Figure 6. On Axis GRIN Lens Imaging. Thorlabs Star target imaged through a GRIN lens with objective aligned on axis (left) and 5° off axis (right). Off axis imaging tilts the image plane, causing intensity gradient across image in tissue due to scattering of deep tissue. Even small angles cause notable gradients.

GRIN Lens Tip Tilt Alignment

Thorlabs recommends adjusting the alignment with widefield single photon imaging (e.g. with a camera) to locate the top surface of the GRIN lens before multiphoton imaging. This is important as focusing the multiphoton laser inside the GRIN lens may etch the surface or interior of the GRIN lens. To align the GRIN lens to the objective, image the top surface of the GRIN lens with a camera (Figure 7). Imperfections in the lens surface ease finding the correct focal plane. As the GRIN lens is typically implanted in an animal, the entire animal needs to be adjusted to bring the GRIN lens into alignment. Here it can be extremely useful for the microscope to rotate in at least one direction, as it is easier to adjust than the animal mount. Thorlabs rotating Bergamo® microscopes are ideal for this purpose as the whole scope and objective rotates about the point of focus, simplifying greatly the adjustments needed to align the scope to the GRIN lens.

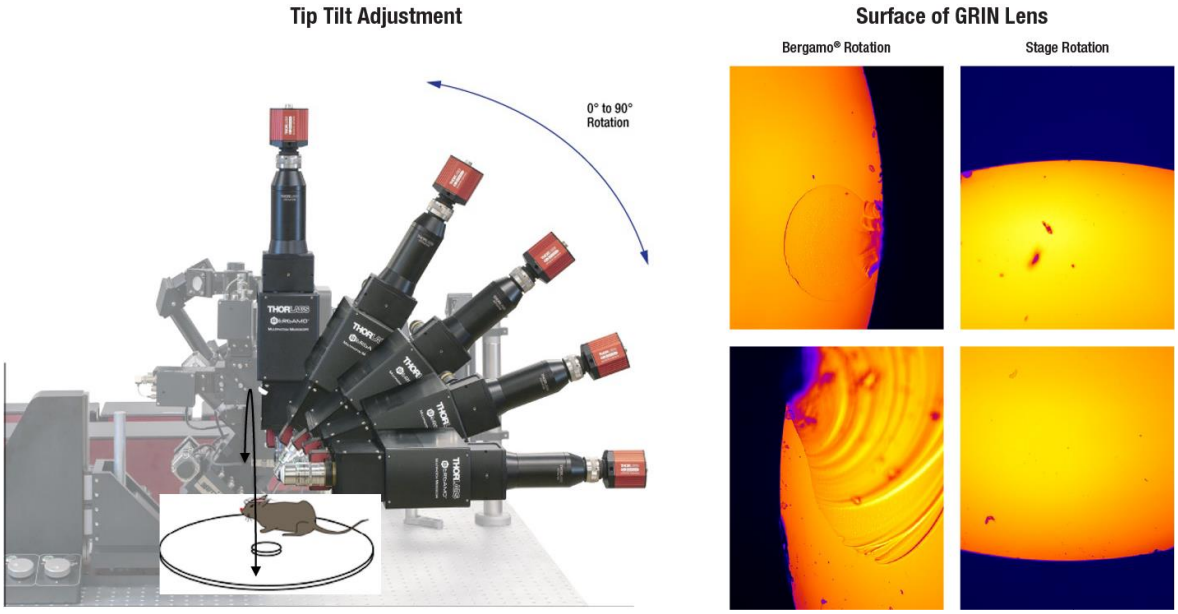


Figure 7. GRIN Lens Alignment. By carefully adjusting the angle of the microscope and the animal stage, the GRIN lens can be exactly aligned with the objective. In this example (left), the GRIN lens is rotated by adjusting the animal in one axis, and the Bergamo® rotation microscope is rotated in the orthogonal axis. Alignment is checked by imaging the top surface of the GRIN lens (right), to make sure all edges are in focus.

Axial Chromatic Aberration in Objectives

It is incredibly important to avoid focusing the pulsed multiphoton laser beam on the GRIN lens surface or inside the GRIN lens as even moderate laser power is enough to etch many GRIN lenses. We recommend that the user mark the focal depth of the GRIN lens surface (e.g., set a zero reference), and make a note to avoid imaging at this depth. Once the GRIN lens is aligned with the camera above, it is recommended to raise the objective by the working distance of the GRIN lens; this will approach the GRIN lens ideal imaging plane.

Because the GRIN lens is not chromatically corrected, the working distance of the GRIN lens will differ with wavelength with longer wavelengths elongating the GRIN working distance ($WD_{GRIN\ sample}$, see Figure 8).

This effect is partially compensated by the objective. Most objectives are overcorrected in the infrared, and therefore focus shallow relative to visible wavelength (shorter WD_{Obj}). Note this shallow focus doesn't change the sample working distance of the GRIN lens, it will still be deeper in infrared relative to visible.

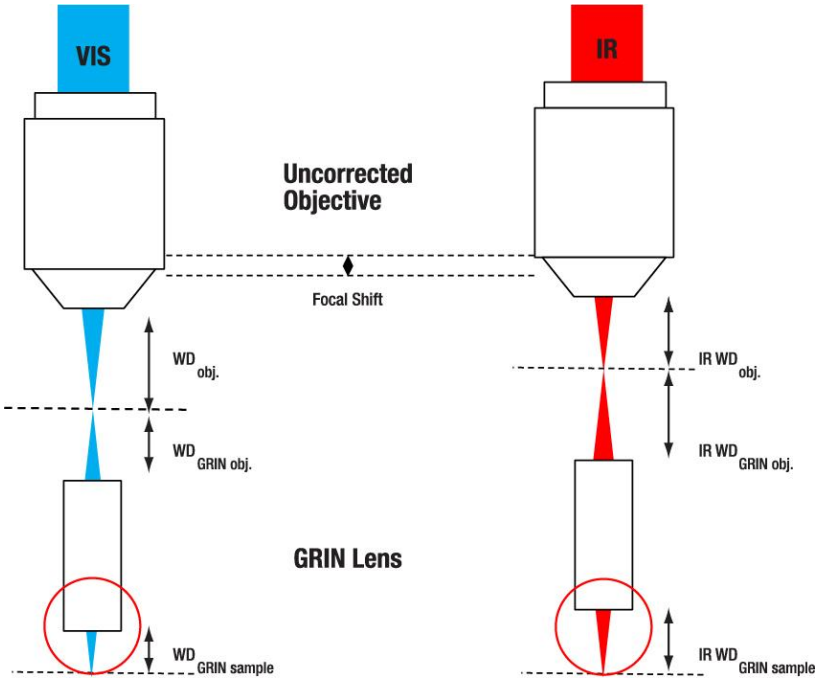
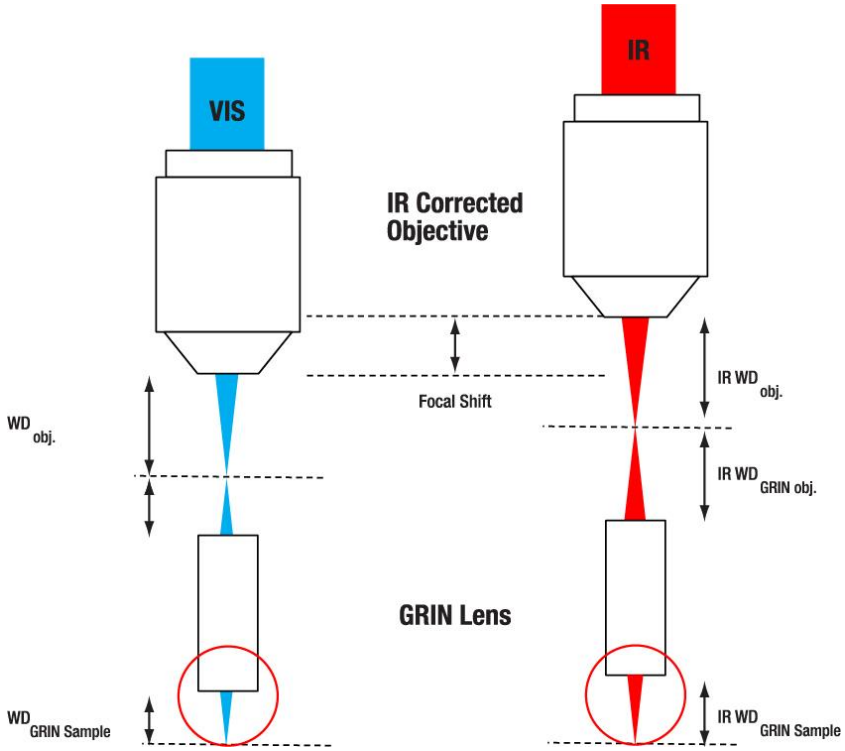


Figure 8. Axial Chromatic Aberration in Objectives and GRIN Lenses. IR uncorrected objectives are generally over corrected in the infrared, i.e. they focus infrared light shallow relative to visible light. Typical GRIN lenses focus longer wavelength light farther from the lens surface. When imaging through a GRIN lens these two effects partially cancel out, and small focus shifts are needed to switch from infrared to visible. Note the GRIN lens sample working distance is still longer in the infrared.



Axial Chromatic Aberration in GRIN

For objectives that are corrected in the infrared, the longer working distance of the GRIN lens is more apparent, and the objective will need to be moved significantly further from the GRIN lens to reach the ideal working focus at the relayed image plane. (see Figure 9).

Figure 9. Axial Chromatic Aberration in Objectives and GRIN Lenses. IR corrected objectives focus IR light and visible light at the same focal plane, this will unmask the GRIN lens working distance change between IR and visible, requiring an upward focal shift of the objective when switching between IR and visible light.

General Considerations

GRIN lenses generally have significant curvature of field (see Figure 10). This field curvature varies from lens to lens, but as it is one design parameter, improving curvature of field results in compromises in other areas. Field curvature, and indeed most aberrations, are related to GRIN pitch, the higher the pitch, the greater the aberrations.

GRIN lenses are designed to image with minimum aberrations at their working distance. However, it is possible to image above or below the designed working distance. Imaging away from the working distance will introduce aberrations into the image. On axis this will result in moderate spherical aberrations [4]. In practice these spherical aberrations are not so apparent in multiphoton imaging, and it is possible to image a significant-sized volume above and below the ideal working distance. However, off axis aberrations increase dramatically, creating issues towards the edge of the field. The most prominent off axis aberration is astigmatism, which significantly degrades GRIN lens FOV away from the ideal working distance [4].

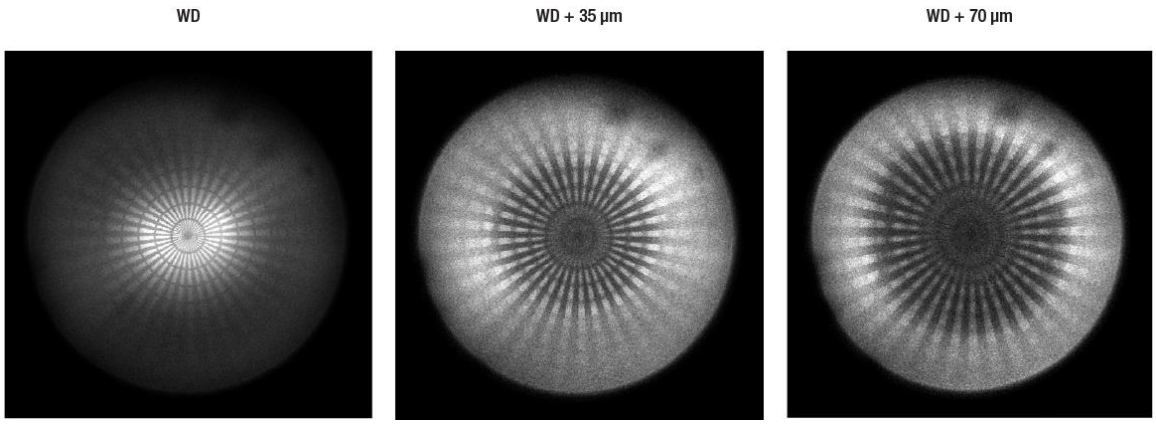
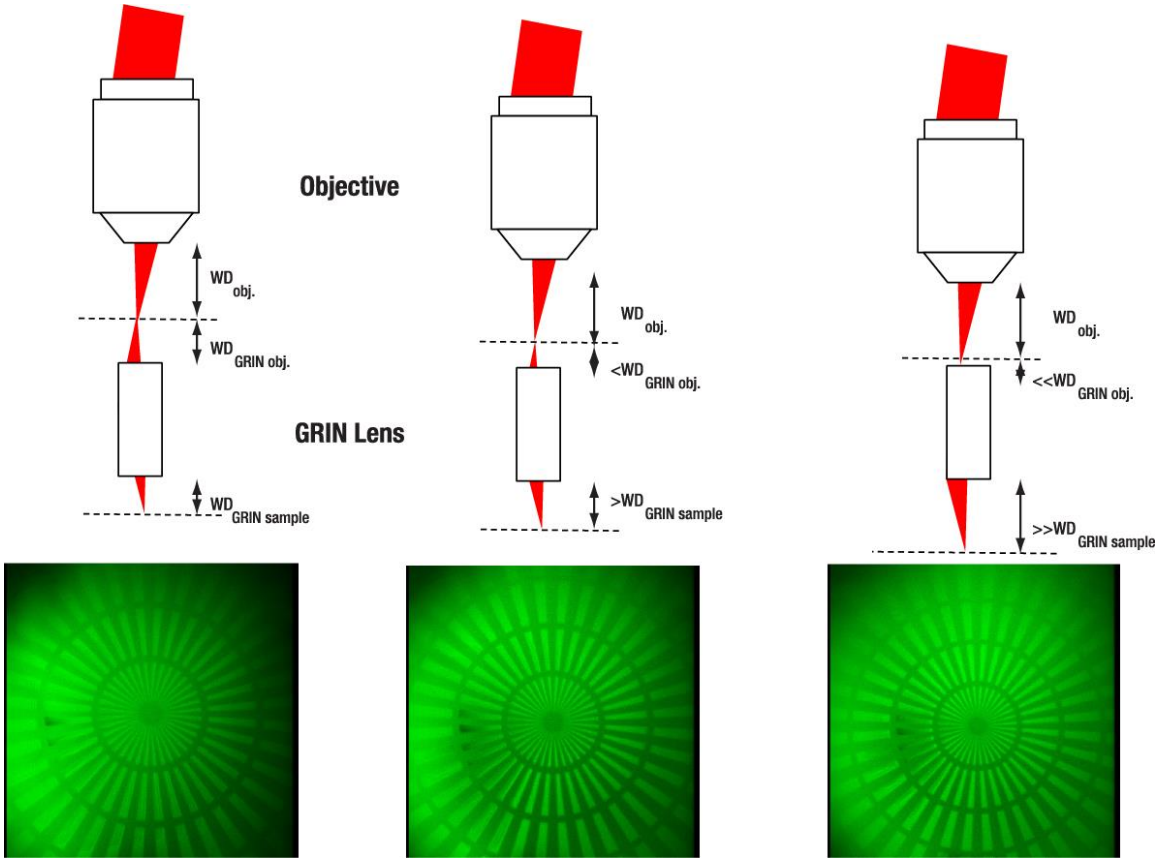


Figure 10. GRIN Lens Field Curvature. Thorlabs Star target imaged through fluorescein and a 1 mm diameter, 9 mm long GRIN lens. This high pitch GRIN lens introduces significant optical aberrations, including field curvature. Since the field curves toward the GRIN lens, one must focus deeper to image the same focal plane off axis WD = Working distance. A flat field would have roughly equal brightness rather than a spot or ring.



Magnification Changes with Focus

In addition to optical aberrations, imaging away from the GRIN lens working distance will change effective magnification. This is because GRIN lenses function as simple lenses: focusing on differing depths changes the effective magnification. This effect can be quite significant, with magnification changing with imaging depth (see Figure 11).

Since system magnification correlates with depth, and imaging depth is uncertain due to chromatic aberrations, care must be taken when assigning magnification, including scale bars. Unless one knows they are imaging exactly at the working distance, there is uncertainty as to the true magnification of the system.

Figure 11. GRIN Lens Magnification and Focus. Changing the focus of the objective relative to the GRIN lens shifts the relayed focus point in tissue. Thorlabs Star targets imagined through a GRIN lens at WD, WD + 80 µm and WD + 160 µm. The farther one focuses through a GRIN lens the lower the system magnification.

Aberration Correction of GRIN Lenses

Because they are simple, uncorrected lenses, GRIN lenses exhibit spherical aberrations, chromatic aberrations, and astigmatism. While these aberrations impact multiphoton imaging less than widefield imaging, the effects are still significant, particularly off-axis and away from the working distance. Over the years various labs have introduced optical elements to reduce these aberrations. Initially, spherical aberrations were minimized with a compound lens design [5], which combined a singlet with a GRIN lens. A compact solution was developed by the Fellin group, which has used aspheres and a coverglass to minimize both on axis and off axis aberrations [6].

An alternative solution is to use adaptive optics wavefront shaping to correct for the aberrations introduced in the GRIN lens [7]. Since multiphoton resolution is dependent upon the excitation pathway, shaping the excitation wavefront serves to improve resolution. This technique more extensively corrects for aberrations in the GRIN lens at the expense of introducing a wavefront modulating device into the imaging path.

Photoactivation and GRIN Lenses

It is possible to combine photoactivation at one wavelength with GRIN lens imaging at another [8]. However, much care must be taken with calibrating the photoactivation focus vs the imaging focus: the axial chromatic aberrations in the GRIN lens, the change in working distance and consequently magnification must be compensated. It is recommended that the photoactivation path be calibrated with the GRIN lens of interest, relative to the imaging pathway.

Spatial light modulators may be combined with GRIN lens imaging to photoactivate multiple cells, in 3D, simultaneously. This multiple cell targeting is best paired with a mechanism to correct for GRIN lens aberrations, for instance via SLM mediated adaptive optics [9], or combined with other optical elements that help mitigate GRIN lens' aberrations. Either way, careful calibration of 3D stimulation is required to correct for zoom and distortion artifacts.

Conclusion

Combining GRIN lenses with multiphoton imaging greatly extends the reach of the system into otherwise highly scattering tissue environments. GRIN lenses maintain multiphoton optical sectioning and can facilitate highly targeted photostimulation experiments with minimal perturbation of deep brain activity. Despite the optical aberrations and complexities arising from the GRIN lens and its alignment with other lens components, the benefits of including a such a GRIN lens setup far outweigh the trouble of including and should be considered when imaging depth is a limitation in experimental design.

Acknowledgements

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To this day, we have a love for science and an eye toward the future. We work hard to design and manufacture the tools that enable the next great steps in scientific discovery.

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