In vivo imaging of unstained tissues using long gradient index lens multiphoton endoscopic systems

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Abstract: We characterize long (up to 285 mm) gradient index (GRIN) lens endoscope systems for multiphoton imaging. We fabricate a portable, rigid endoscope system suitable for imaging unstained tissues, potentially deep within the body, using a GRIN lens system of 1 mm diameter and 8 cm length. The portable device is capable of imaging a ~200 µm diameter field of view at 4 frames/s. The lateral and axial resolution in water is 0.85 µm and 7.4 µm respectively. In vivo images of unstained tissues in live, anesthetized rats using the portable device are presented. These results show great promise for GRIN endoscopy to be used clinically.

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1. Introduction

Two-photon fluorescence (TPF) and second-harmonic generation (SHG) microscopy are powerful tools for imaging unstained biological tissues [1]. These label-free techniques are capable of producing high-resolution real-time in vivo images and have shown great promise for medical diagnostics of various diseases, potentially replacing surgical biopsies [2–7]. The maximum imaging depth, however, is limited in most tissues to ~1 mm [8,9].

One strategy to overcome the depth limitation is to develop miniaturized TPF microscopes that could be used as endoscopes in a clinical setting. A number of different endoscopes and techniques have been demonstrated [10–17], including in vivo imaging of unstained tissues [18]. These devices are typically composed of a miniaturized scanning mechanism and focusing optics in a protective housing. The scanners proposed generally use either a miniaturized fiber scanning mechanism or microelectromechanical systems scanning mirror. The need to encapsulate a scanning mechanism into a housing of suitable size for minimally invasive procedures poses several challenges including: (1) uniformity of scan, (2) sensitivity, durability and reliability of the scanner, and (3) miniaturization of the distal scan mechanism and optics. A different approach has been to use gradient index (GRIN) lenses to relay the excitation light and TPF/SHG emission to and from an external microscope deep into soft tissue [19–22]. Since only the GRIN lens penetrates the tissue, the excitation, scanning, and collection optics need not be miniaturized for in vivo imaging. GRIN lenses have been shown to be biocompatible and previously used in a clinical setting for non-penetrative imaging of

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chronic leg ulcers with delayed wound healing [23]. Use of a small diameter lens to penetrate deeply within tissue has been demonstrated with a hypodermic needle GRIN system [24]. These studies show great promise for GRIN endoscopy to be used as either a guide for or a replacement of traditional surgical biopsies.

Previous studies, however, have been limited to short (<4 cm) GRIN systems primarily intended for small animals and external clinical use [19–23]. The eventual adaptation of GRIN lenses in the diagnosis of human diseases would require significantly longer GRIN endoscope systems. Commonly used prostate biopsy needles, for example, are as long as 25 cm. In this study we investigate the effect of GRIN system length on the imaging performance. We characterize the loss in imaging performance with increased GRIN system length and present a compact and portable two-photon microscope that could be used in a clinical environment in combination with various GRIN lens systems. Finally, the capability of the system for in vivo imaging of unstained tissues is demonstrated in live, anesthetized rats.

2. GRIN endoscopes

GRIN lenses use a negative gradient in the refractive index of glass with increasing radius to bend and focus light. The pitch of a GRIN lens characterizes how many internal images are formed within the lens, with a pitch of 1 being the length of one full sinusoidal path. Most previously reported GRIN two-photon endoscope systems use a combination of relay and objective GRIN lenses in either a doublet (relay/objective) or triplet (objective/relay/objective) system [19–23]. The usually low NA relay lens avoids a tight focus of the excitation light within the glass, while the higher NA objective lens allows for high-resolution two-photon imaging at the sample. Previous studies have shown that a doublet system offers several advantages over a triplet system such as similar fields of view at reduced lens cost, greater image magnification, lower NA for coupling the laser beam, and lower autofluorescence at the endoscope surface [21]. GRIN lenses are commercially available in varying lengths and in diameters as small as 350 µm. We obtained doublet systems of 1 mm and 2 mm diameter from GRINTECH GmbH consisting of a 0.5 NA objective lens and a 0.1 NA relay lens of varying length and pitch. The different systems obtained are summarized in Table 1. All systems were designed for 800 nm excitation light and with working distances of 100-140 µm in air on either side (~140-190 µm in water). The GRIN lens systems were protected by a stainless steel tube of 0.1 mm thickness, resulting in total system outer diameters (OD) of 1.2 mm and 2.2 mm.

| Table 1. Summary of Optical Characterization Results* |
|-----------------------------------------------|
| **GRIN System** | **Part Number** | **Diameter (mm)** | **Length (mm)** | **Relay Lens Pitch** | **FWHM (µm)** | **Diameter of FOV (µm)** |
|----------------|-----------------|------------------|----------------|----------------------|---------------|-------------------------|
| 1A             | GT-ERLS-100-075-11-50-NC | 1.00             | 35.9           | 0.75                 | 0.94          | 10.8                    | 199                     |
| 1B             | GT-ERLS-100-175-11-50-NC | 1.00             | 79.9           | 1.75                 | 0.97          | 12.0                    | 195                     |
| 2A             | GT-ERLS-200-075-11-50-NC | 2.00             | 81.4           | 0.75                 | 0.99          | 12.6                    | 370                     |
| 2B             | GT-ERLS-200-125-11-50-NC | 2.00             | 132.6          | 1.25                 | 1.05          | 15.6                    | 365                     |
| 2C             | GT-ERLS-200-275-11-50-NC | 2.00             | 285.0          | 2.75                 | 1.17          | 25.0                    | 359                     |

*Five different doublet GRIN systems were designed by and purchased from GRINTECH GmbH. The lateral and axial two-photon resolutions were determined in air using subresolution fluorescent beads and a thin rhodamine film respectively.

3. Experimental setup

Figure 1 shows the custom built horizontal multiphoton microscope used to characterize the GRIN lens systems. A mode-locked Ti:sapphire laser (Tsunami, Spectra Physics, Inc.) was

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used as the excitation source at 800 nm with 10 nm bandwidth. Dual axis (5 mm diameter) galvo based scan mirrors (GVSM002, Thorlabs Inc.), and two scan lenses of 10 cm and 30 cm focal length (respectively, AC508-100-B-ML and AC508-300-B-ML, Thorlabs Inc.) were used to scan the beam angle at the overfilled back aperture of a 0.1 NA microscope objective. The focus of the objective was raster scanned by the galvo mirrors across the proximal face of the GRIN lens systems. The GRIN lens systems were mounted on a three axis manual linear translational stage close to the focal plane of the objective to aid alignment. All optical characterizations were conducted by moving the sample mounted on a 3D stage (MP-285, Sutter Instruments) allowing axial scanning of the sample while maintaining the GRIN lens system in a fixed position. The fluorescent signal from the sample was epi-collected through the GRIN lenses and the microscope objective. Collected light is reflected by a dichroic beam splitter (FF-665-Di01, Semrock Inc.). After passing through two 575/250 bandpass filters (HQ575_250 2p, Chroma Technology Corp.) separated by a colored glass (FGS900, Thorlabs Inc.), the fluorescence is detected by a photo-multiplier tube (PMT) (HC125-02, Hamamatsu Photonics). Data acquisition and motion control were implemented using a DAQ card (PCI-6115, National Instruments Corp.) and MPScan software [25]. The axial resolution of the GRIN lens systems was characterized in air using the full width at half maximum (FWHM) two-photon excited fluorescence signal from a 500 nm thin film of Rhodamine B (RhB) dye, while the lateral resolution was characterized using FWHM two-photon excited fluorescence from subresolution (0.2 µm) fluorescent beads. The field of view (FOV) was characterized by raster-scanning the proximal face of the GRIN lens system and measuring the one-photon transmission using a photodiode (SM05PD1A, Thorlabs Inc.), and was defined as the FWHM of the resulting intensity profile.

4. GRIN endoscope characterization

The on-axis lateral and axial resolutions and FOV of the systems are summarized in Table 1. Figure 2 shows the beam profile along the axial and lateral directions of the longest GRIN system (2C). Increasing the length of the relay lens results in only a small deterioration of the lateral resolution. The axial resolution declines more significantly for the longer systems. We believe that this is mainly due to increased accumulation of spherical aberrations, as can be seen by the asymmetric thin film response curve in Fig. 2(b). These characterizations were done in air; we would expect the axial resolution scaled by a factor of ~1.3 in tissue. Nevertheless, axial resolution remains on the order of one layer of mammalian cells for all of
the GRIN lenses tested. The FOV for all systems (195-370 μm) is relatively large for the small OD systems. Figure 3 shows the most and least uniform intensity curves obtained from the transmission imaging (GRIN system 1B and 2C respectively).

The off-axis axial resolutions of the different systems were measured by acquiring a through-focus z-series of the RhB thin film and fitting a Lorentzian function to individual off-axis areas of ~6 by 6 μm in size. The resulting FWHM of each area is plotted in Fig. 4. The lateral off-axis performance was also measured for system 1B using sub-resolution fluorescent beads, and the FWHM of the obtained PSF remained below 1.2 μm up to the edge.
These results indicate that the off-axis resolution of these GRIN systems remains within ~20% of the on-axis resolution for most of the FOV (~80% of the area). The ability to deliver ultrashort pulses to the sample is critically important for multiphoton imaging. The effect of the longest GRIN lens system (285 mm in length, system 2C) on the excitation laser pulse was characterized. Without dispersion compensation, the initial 80 fs pulse width was broadened to 740 fs. Using precompensation with a rotating cylindrical lens and grating [26], we were able to achieve an 85 fs pulse width at the sample, indicating that a simple dispersion compensation setup that accounts for the second order dispersion is sufficient for delivering pulses on the order of 80 fs.

5. Portable endoscope design and system characterization

To demonstrate that these GRIN lens systems have potential clinical applications, a compact, fiber-coupled multiphoton GRIN lens endoscope was constructed for in vivo image acquisition (Fig. 5). The 800 nm femtosecond pulse from the Ti:sapphire laser is delivered by a 2 meter hollow core PCF fiber (HC-800B, Thorlabs Inc.), and collimated to a beam of about 2 mm diameter using an aspheric lens. A small aperture (3 mm) galvo scanning mirror system (6210H, Cambridge Technology) was selected for a fast imaging rate (up to 4 frames/s at 512 by 512 pixels). The beam is then expanded by two scan lenses of 18 and 36 mm focal length (respectively, LSM02-BB and LSM03-BB, Thorlabs Inc.) to underfill a 0.3 NA microscope objective (RMS10X-PF, Thorlabs Inc.) to achieve an effective NA of ~0.1. The microscope objective couples the excitation beam into the proximal side of the GRIN lens system. We selected the longest 1 mm diameter system (1B) for this demonstration. The fluorescence signal from the sample is epi-collected through the GRIN lenses and the objective, and is reflected by a dichroic beam splitter (FF705-Di01, Semrock Inc.). After passing through two short pass filters (FF01-720/SP, Semrock Inc.) separated by colored glass (FGS900, Thorlabs Inc.), a second dichroic (Di01-R405, Semrock Inc.) separated the signal into the second harmonic and the autofluorescence channel. The housing of the GRIN lens endoscope was constructed from custom machined aluminum components using a milling machine with a fabrication tolerance of 0.001". Optical elements in the GRIN endoscope were aligned in the z-axis to specification using calipers. We verified that illumination light projected through the system was centered as it passed through each optical element. The GRIN lens position was verified by confirming the focal length of the lens met specification (i.e. verified GRIN lens working distance by locating peak two-photon excited signal of a fluorescent sample ~130µm

Fig. 5. Portable GRIN endoscope. (a) Optical drawing and (b) Solidworks drawing of the GRIN based endoscope system. Total system length of the portable device is 10.6" (including GRIN system).
from the lens using a three-axis micrometer). The hollow core fiber has anomalous dispersion at 800 nm. This allowed us to precompensate the dispersion of the fiber and the GRIN lens by using a 10 cm rod of dispersive SF10 glass in the beam path before coupling into the fiber, resulting in a ~120 fs pulse width at the sample. The axial resolution of the GRIN lens endoscope system is 6.5 μm FWHM in air and 7.4 μm FWHM in water, measured using the RhB thin film as described above. The measured lateral resolution in both air and water is 0.85 μm FWHM using sub-resolution beads.

Fig. 6. Unaveraged *in vivo* images of unstained rat tissue. The pseudo-color images show red SHG signal (<405 nm) and green intrinsic fluorescent emission (405-700 nm). (a) Image of the superficial kidney renal cortex shows dark renal interstitium (RI), dark cellular nuclei (N) and bright intrinsic fluorescent cytoplasm (CY) that form the epithelial cells in the renal tubules (RT), SHG signal from the tough fibrous layer that forms the renal capsule (RC), and the dark blood filled lumen (L) inside the renal tubules. (b) Image of the inner colon wall shows bright intrinsic fluorescent signal from enterocytes (E) surrounding dark circular crypts (C). (c) Image of the rat liver showing ~20 µm diameter hepatocytes (coarse dashed line) with dark nuclei (N, solid line) chained together to form hepatic chords (HC), a dark bile duct (BD, fine dashed line) and bright intrinsically fluorescent bile salts (BS), as well as SHG emission from the septa (S) a fibrous tissue bands that separates hepatocyte nodules. (d) Image of the rat liver without labels shown for clarity. In these images, scale bars are 20 µm.
6. In vivo imaging

The ultimate goal of multiphoton endoscopy is imaging human patients in a clinical environment. A critical intermediate step is imaging unstained, live animals. Here, we demonstrate the capability of the portable GRIN endoscope in in vivo imaging of unstained tissues in live rats. A male rat (250-350 grams, Sprague-Dawley, Charles River Laboratories International, Inc.) was anesthetized using a gas anesthetic (~5% isoflurane-oxygen mixture) in an induction chamber. After reaching the appropriate level of sedation for surgery, the animal was fitted with a nose cone to maintain sedation (~2-3% isoflurane-oxygen mixture). Rat body temperature was maintained with a feedback-controlled heat blanket set to 36 °C. A ventral-midline abdominal incision exposed the internal organs to the GRIN endoscope. The kidney and liver were isolated and elevated using tongue depressors, and the surfaces of these organs were imaged using the portable GRIN endoscope. Another incision was made into the colon to expose and subsequently image the inner lining of this organ.

All animal procedures were reviewed and approved by the Cornell Institutional Animal Care and Use Committee. Throughout imaging sessions, the portable GRIN endoscope was mounted on a flexible mechanical arm (18041-XL-Special, Flexbar Machine Corporation), which allowed for rapid coarse movement of the endoscope from organ to organ. The flexible arm was in turn mounted on a 3D stage (MP-285, Sutter Instruments) for fine movement control. Images were acquired at a rate of 4 frames/second without averaging and with approximately 75mW at the sample. Example images are shown in Fig. 6, pseudocolored with autofluorescence in green and second harmonic signal in red. Motion artifacts present a major challenge for in vivo imaging experiments. Most of the observed artifacts were due to respiratory motion, and seemed to increase the closer the imaged organ was to the diaphragm. Most of the images of the colon (>90%) are of comparable quality to those shown in Fig. 6, and this was true for ~70% of the kidney images. During imaging of both these organs, respiratory rate of the animal was at around 45 breaths per minute. The liver images suffered the most from motion artifacts, with only ~65% of similar quality as Fig. 6 at 45 breaths per minute. Once respiratory rate was decreased temporarily to ~25 breaths per minute by increasing the isofluorane:oxygen ratio in the gas anesthetic, the amount of high-quality images increased to ~80%. In comparison, we note that the respiratory rate for a healthy human adult is approximately 12-20 breaths per minute.

7. Discussion

Both the axial and lateral resolution obtained for our systems (1A, 1B, 2A, and 2B) are similar to previous reports of similar NA systems at much shorter lengths [19,20,22,23]. Our longest system, 2C, shows a significant degradation in axial resolution. This is probably due to the accumulation of spherical aberrations and manufacturing imperfections with increasing relay lens length. The lateral resolution, however, is only minimally affected, and the axial resolution remains on the order of a layer of mammalian cells, showing promise that long GRIN endoscopy has diagnostic potential. This is also evident from our in vivo imaging results. While image quality is decreased slightly when compared to a microscope objective of similar NA, many of the important tissue features are identifiable, show similarity to anatomic features seen in histology slides, and are potentially useful for diagnosis [27]. It is also noteworthy that no tissue damage was observed throughout the in vivo experiments, and our imaging conditions were comparable to those previously shown to have negligible tissue mutagenicity [28,29]. In our imaging experiments, using up to 75mW illumination power at the sample, we did not witness any laser-induced visible luminescence within the GRIN endoscope, a concern with increasing relay lens length due to the increased number of internal foci. Previous studies have found this effect in higher NA systems (0.45 NA) with a threshold of 73 mW at 800nm [23]. We did observe, however, when the GRIN lens was misaligned to the extent that the distal focus was inside the high NA GRIN objective lens, the endoscope
probe was damaged. The FOV for all systems remained large enough for diagnostic potential (195-370 μm diameter).

While long GRIN lens systems are limited to rigid endoscope applications, they offer several advantages over flexible multiphoton endoscopes. A significant advantage is the diameter of the endoscope probe. GRIN lenses are commercially available in diameters as small as 350 μm, which allows them to be inserted into needles as small as 22 gauge (inner diameter of 413 μm). Using the doublet design, we would expect similar imaging performance with such a lens except with a smaller FOV of about 70 μm diameter. Furthermore, the use of externally mounted galvo based scanning mirrors solves several challenges faced by other endoscope designs when considering clinical implementation such as uniformity of the scan and durability of the device. Because GRIN lenses are inexpensive, they could ultimately be used as disposable devices, eliminating the need for sterilizing the endoscope probe between procedures. The GRIN lens approach would also allow for an external focus adjustment in the depth of the sample without movement of the endoscope probe that has penetrated the tissue. By axially translating the scan objective, a z-scan in the sample of 0 to 95 μm from the surface of the lens has previously been shown [20].

8. Conclusions

We have demonstrated that TPF and SHG imaging are possible through long GRIN lens systems up to 28.5 cm in length. Long GRIN lenses can be integrated with a compact and portable two-photon microscope suitable for a clinical environment. The device presented can acquire TPF and SHG images at a rate of 4 frames/s with a field of view of ~200 μm diameter and with subcellular resolution. The presented in vivo results of unstained organs of live rats show great promise for using GRIN endoscopy for optical biopsy.

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