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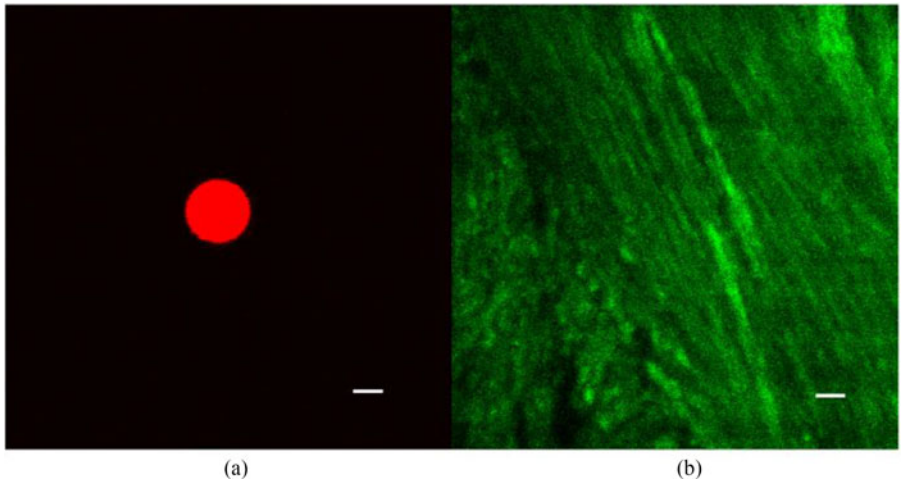

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Transmittance Characterization of Objective Lenses Covering all Four Near Infrared Optical Windows and its Application to Three-Photon Microscopy Excited at 1820 nm

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Abstract: Near infrared (NIR) excitation or emission is capable of deep-tissue penetration in various modalities of optical microscopy. Based on the transmittance characterization of biological tissue, such as brains, four NIR optical windows have been demonstrated or suggested: The 800-nm, the 1300-nm, the 1700-nm, and the 2200-nm window. High-numerical aperture objective lenses are needed in optical microscopy to both deliver sufficient excitation light and collect efficient signal light to enable deep-tissue imaging. The transmittance performances of objective lenses are of vital importance. However, there is a lack of experimental characterization of the transmittance, especially at long wavelengths, which poses a dramatic obstacle for lens selection in imaging experiments. Here, we demonstrate detailed measurement results of the transmittance performance of air, water-immersion, and oil-immersion objectives available to us, covering all the four NIR optical windows. These results will provide direct guidelines for objective lens selection in terms of transmittance performance. We further demonstrate three-photon microscopy with 1820-nm excitation, close to the edge of the 1700-nm window, using the objective lens based on our transmittance measurement.

Index Terms: Fluorescence microscopy, nonlinear microscopy, multiphoton processes.

1. Introduction

Optical microscopy has enabled visualization of subcellular structures and always been an indispensible tool for a broad range of applications including medical and life sciences. Traditional optical microscopy excites (or illuminates) samples and detects signals at the optical wavelength, ranging from ultraviolet to near infrared (NIR). Compared with excitation at shorter wavelengths, excitation or detection at NIR takes advantage of the smaller scattering due to the longer wavelength, especially for highly inhomogeneous biological tissues. So far, NIR excitation and detection has been applied

to deep-tissue one-photon [1] or multiphoton microscopy (MPM) [2]–[6], pushing the imaging depth to new levels.

Based on both the absorption (mainly water, the main composition of most soft tissues) and scattering characteristics of biological tissues, several NIR optical windows have been suggested and experimentally verified for deep-tissue imaging. These include the 800-nm window, the 1300-nm window and the 1700-nm window [7], [8]. Taking in-vivo mouse brain MPM for example, the 800-nm window [2], [5], the 1300-nm window [3], [6] and the 1700-nm window [4] have seen increasing imaging depth as the excitation wavelength increases. Although haven't demonstrated yet, based on the water absorption spectrum measurement [9] and tissue transmittance measurement [8], it is suggested that the 2200-nm window is also a potential NIR optical window for deep-tissue imaging.

To achieve subcellular resolution, high numerical aperture (NA) objective lenses are commonly used in optical microscopy. While other optical performances of the objective lenses have been extensively investigated to achieve the best imaging results, the transmittance performance and data are quite scarce [10], [11]. To the best of our knowledge, there are no measured results covering all the four NIR optical windows. This is because most of the objective lenses are designed and coated for the visible, the 800-nm and the 1300-nm windows only. As a result, even the manufacturer themselves cannot provide the transmittance data covering all four NIR optical windows upon request. This dramatically hampers the selection of objective lens, especially when imaging experiments are targeting the 1700-nm and the 2200-nm window. As end users, it is almost impossible to purchase a large number of and often quite costly objective lenses for test, just for the sake of selecting the one with the best transmittance performance. The transmittance of the objective lens is, however, one of the key parameters to consider and optimize when long NIR wavelengths are involved. For example, in 3-photon microscopy, a 2-fold lower transmittance of the objective lens will result in an 8-fold decrease in 3-photon signal.

It is our main aim in this paper to provide extensive measured transmittance data for a variety of high NA objective lenses commonly used in microscopy, covering all four NIR optical windows. The total eight measured objective lenses available to us encompass air, water-immersion and oil-immersion. In addition to the measured broadband transmittance curves, we also list the transmittance data of these objective lenses at 800 nm, 1300 nm, 1700 nm and 2200 nm. Based on these measured results, we further demonstrate 3-photon microscopy using 1820-nm excitation, longest excitation ever demonstrated at the 1700-nm window. We believe these results will facilitate objective lens selection for various modalities of optical microscopy. We note that other NIR wavelengths not falling within these windows, such as 1040 nm [12] and 1550 nm [13], may also benefit from these measurement.

2. Experimental Setup and Sample Preparation

2.1 Transmittance Measurement of the Objective Lenses

Table 1 lists the eight objective lenses measured and available to us in this paper, including details such as model, NA, working distance (WD), etc. For transmittance characterization of the objective lenses covering visible and all four NIR optical windows, a broadband spectrometer (Lambda 900, Perkin Elmer) equipped with an integrating sphere (PELA 1000, Perkin Elmer) were used. The integrating sphere is necessary to collect the large divergent rays after the high NA objective lenses. Similar to that in [10], a pinhole was placed in front of the objective lens to narrow the incoming beam from the spectrometer, so that all rays would be transmitted to the integrating sphere except those reflected or absorbed by the objective lens. For all these objective lenses, we measured a broadband range covering 350 nm to 2500 nm.

2.2 Laser and Imaging Setup

So far at the 1700-nm window, only wavelengths below 1700-nm have been demonstrated for MPM [4], [14], although it has been suggested the entire window covers up to 1850-nm [15], [16],

TABLE 1
List of the Eight Objective Lenses Measured

Model	NA	WD (mm)	Immersion	Magnification	Manufacturer
UPLSAPO 40X2	0.95	0.18	Air	40x	Olympus
UPLFLN 40X	0.75	0.51	Air	40x	Olympus
N PLAN	0.65	0.36	Air	40x	Leica
XLPLN25XWMP2-SP1700	1.05	2	Water	25x	Olympus
XLPLN25XWMP2	1.05	2	Water	25x	Olympus
XPLN25XSVMP2	1	4	Water/oil	25x	Olympus
UPLSAPO 30X SIR	1.05	0.8	Oil	30x	Olympus
UPLSAPO 60XO	1.35	0.15	Oil	60x	Olympus

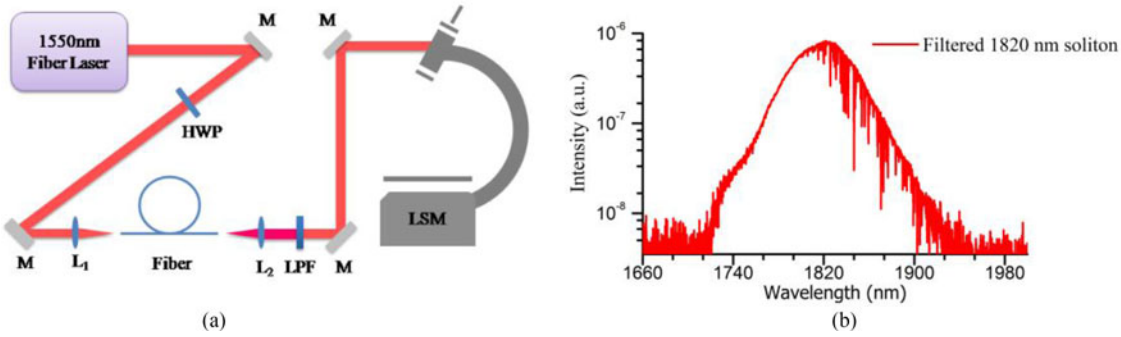

Fig. 1. Experimental setup for SSFS and MPM at 1820 nm (a). M: silver-coated mirror, HWP: half-wave plate, L: lens, LPF: long-pass filter, and LSM: laser scanning microscope. (b) Measured 1820-nm soliton after the LPF.

beyond which water absorption quickly increases [9]. As a practical application of our objective lens measurement, we demonstrated both 3-photon fluorescence and third-harmonic generation (THG) imaging excited at 1820-nm, close to the edge of the 1700-nm window. To generate excitation at 1820-nm, we performed soliton self-frequency shift (SSFS) in 113-cm large mode area (LMA) fiber (DC-200/40-PZ-Si, NKT Photonics). The fiber was pumped by a 1-MHz, 500-fs 1550-nm fiber laser (FLCPA-02CSZU, Calmar). A half-wave plate was used before coupling into the fiber since the fiber was polarization maintained. A 1725-nm long-pass filter (1725LP, Omega Optical) was used to remove the residual pump, leaving only the 1820-nm soliton for imaging. The measured spectrum of the soliton is shown in Fig. 1(b), which was measured using an optical spectrum analyzer (OSA203B, Thorlabs).

The 1820-nm soliton pulses were then sent into a laser scanning microscope (MOM, Sutter) for 3-photon imaging. Two samples were imaged: Crimson fluorescent beads (F8831, Thermofisher) embedded in agarose were used for 3-photon fluorescence imaging and a mouse brain slice was used for THG imaging. Animal procedures were reviewed and approved by Shenzhen University. A GaAsP photomultiplier tube (PMT, h7422p-40, Hamamatsu) with high sensitivity [17] was used to detect the 3-photon signals for both imaging modalities. An amplifier unit (C7319, Hamamatsu) was used for current-to-voltage conversion. A 643/20 bandpass filter (FF01-643/20-25, semrock) and a 605/70 bandpass filter (ET605/70m, Chroma) were used in front of the PMT for 3-photon fluorescence and THG imaging, respectively. Image acquisition and processing were performed using ScanImage (Vidrio Technologies) and ImageJ (NIH), respectively.

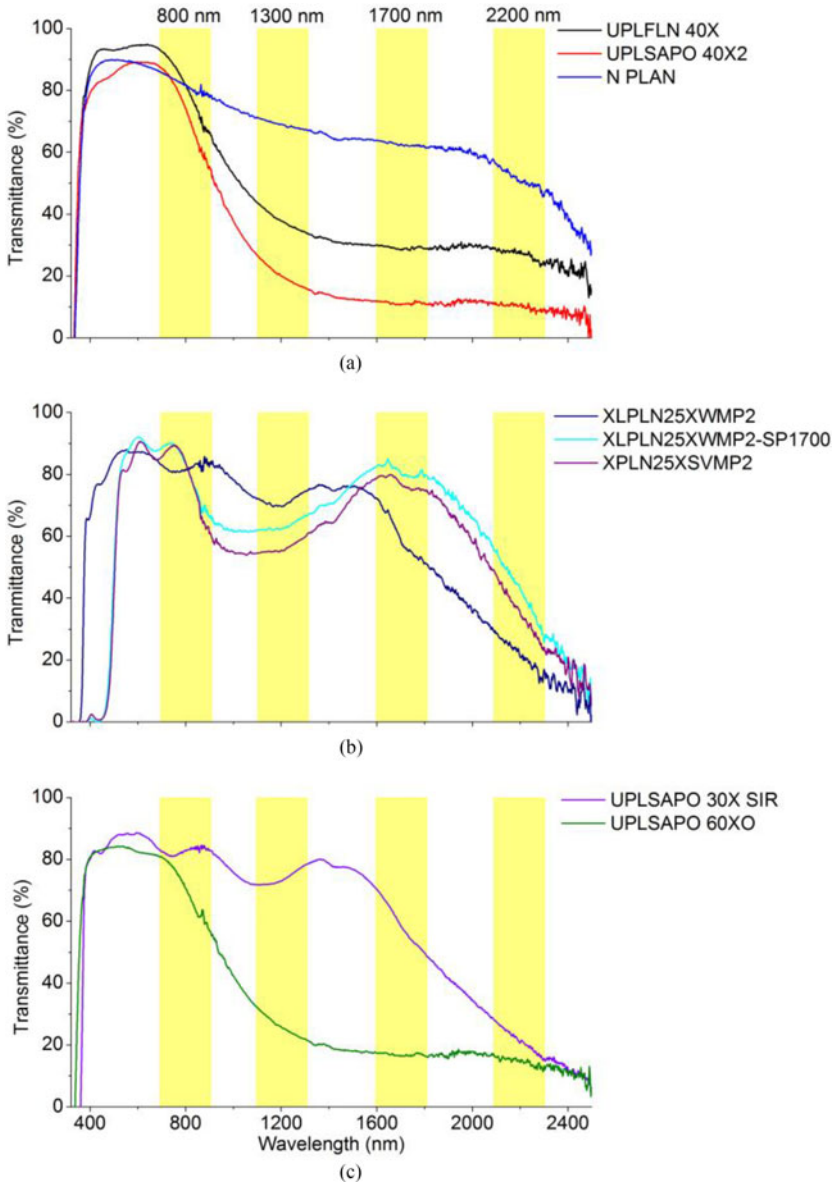

Fig. 2. Measured transmittance of the (a) air, (b) water-immersion (a water/oil immersion included), and (c) oil-immersion objective lenses. Rectangles indicate the four NIR windows.

3. Experimental Results

3.1 Transmittance Characterization of the Objective Lenses

First we measured the broadband transmittance of all the eight high NA objective lenses. The objective lenses are categorized into air, water immersion (the water/oil objective lens is also listed in this category) and oil immersion, and the measured results are shown in Fig. 2(a)–(c), respectively. To highlight the transmittance in the four NIR optical windows, four rectangles corresponding to the wavelength spans of these windows are also shown, covering 650 nm to 950 nm (800 nm window), 1100 nm to 1350 nm (1300 nm window), 1600 nm to 1850 nm (1700 nm window), and 2100 nm to 2300 nm (2200 nm window).

TABLE 2
Transmittance data at 800 nm, 1300 nm, 1700 nm, 1820 nm and 2200 nm

Model	800 nm (%)	1300 nm (%)	1700 nm (%)	1820 nm (%)	2200 nm (%)
UPLSAPO 40X2	74.2.	16.3	10.6	11.2	10.5
UPLFLN 40X	81.9	34.2	28.4	28.7	27.2
N PLAN	81.7	67.3	62.6	61.5	50.1
XLPLN25XWMP2-SP1700	83.2	66.7	80.2	78.5	43.2
XLPLN25XWMP2	81.5	74.6	58.5	49.8	20.9
XPLN25XSVMP2	83.5	59.8	76.8	73.6	35.1
UPLSAPO 30X SIR	83.0	78.2	58.5	47.9	20.6
UPLSAPO 60XO	70.5	22.0	16.5	16.7	14.4

Table 2 further summarizes the transmittance data at 800 nm, 1300 nm, 1700 nm, 1820 nm and 2200 nm, to facilitate direct comparison in terms of transmittance.

Among all the three air objective lenses, the N Plan provides the best overall transmittance over all the four NIR windows. Although the transmittance of N Plan at the 800-nm window is not the best, it provides rather descent transmittance performance at the 1300-nm window (67.3% at 1300 nm), 1700-nm window (62.6% at 1700 nm) and even 2200-nm window (50.1% at 2200 nm). However, compared with the other two air objective lenses, N Plan is lower in nominal NA, which means the resolution is poorer.

For subcellular deep-tissue imaging such as brain imaging, long working distance, water immersion objective lenses are commonly used to better match the refractive indices [4], [6]. For the three water-immersion objective lenses we measured, XLPLN25XWMP2 is a regular one. Both XLPLN25XWMP2-SP1700 and XLPLN25XSVMP2 are customized with special coating for high transmittance for the 1700-nm window. Besides, they are both specifically coated to guarantee high transmittance from 533 nm to 750 nm, which also can be seen from the measured results in Fig. 2(b), in order to provide high signal transmittance for both 3-photon fluorescence from red fluorophores and THG signals excited at the 1700-nm window. Specifically, 3-photon fluorescence can visualize blood vessels and neurons, while THG can visualize the white matter in the brain [4]. According to the manufacturer, XLPLN25XWMP2-SP1700 is a replica of XLPLN25XWMP2 except for the coating. XLPLN25XSVMP2 has the longest working distance of its kind (WD $=$ 4 mm). The regular XLPLN25XWMP2 objective lens has excellent transmittance performance from 380 nm to 1600 nm, beyond which the transmittance decreases rapidly as the wavelength increases. In contrast, customized coating of both XLPLN25XWMP2-SP1700 and XLPLN25XSVMP2 shift the high transmittance plateau to the 1700 nm window, making them ideal choice for deep-tissue imaging such as brain imaging at this window. Another benefit of this shift is that the transmittances of these two objective lenses at the 2200-nm window are also reasonable (43.2% and 35.1%), much better than the regular XLPLN25XWMP2 (20.9%). However, this raised transmittance at the 1700-nm and 2200-nm comes at the cost of reduced transmittance at the visible and the 1300-nm window. The most notable difference is on the visible side below 500-nm, where the objectives with custom coatings virtually do not transmit light. So care should be given if detected signal falls within this range.

When biological tissue with dramatically higher refractive index than water, such as the skin, is to be imaged, oil-immersion objective lenses are commonly used to minimize aberration [18]. The last two objective lenses we measured are exclusive oil-immersion objective lenses, shown in Fig. 2(c). UPLSAPO 30X SIR features a long working distance of 800 μm, and shows much better transmittance over all the four NIR optical windows compared with the UPLSAPO 60XO, especially at the 1300-nm and 1700-nm window. The working distance of UPLSAPO 60XO is too short (WD $=$ 0.15 mm) for deep-tissue imaging.

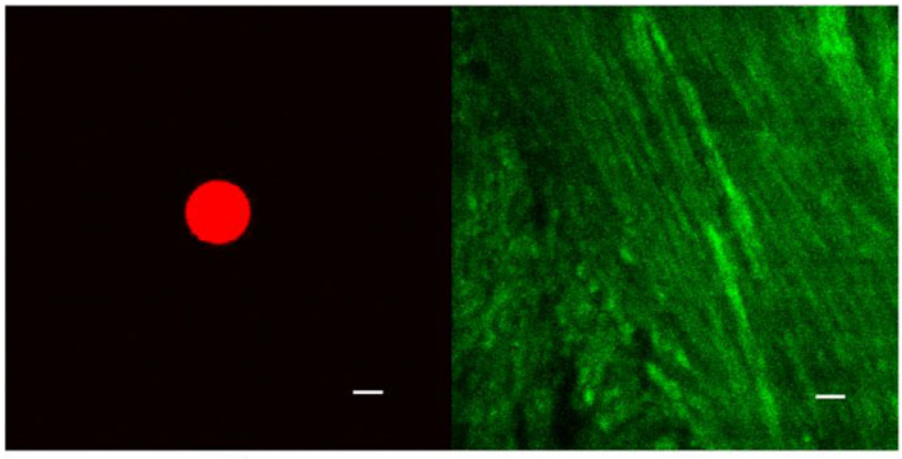

Fig. 3. 3-photon fluorescence imaging of the crimson bead (a) and THG imaging of the mouse brain slice (b), excited by 1820-nm solitons. Scale bar: 10 μm; pixel size: 512 $\times$ 512; frame rate: 2 s/frame for (a) and 8 s/frame for (b). Each image is an average of 2 frames.

3.2 3-Photon Microscopy Excited at 1820 nm

Based on the measured results summarized in Table 2, we can easily find that the customized objective lens XLPLN25XWMP2-SP1700 has the highest transmittance at 1820 nm. Besides, it has high transmittance at both the 3-photon fluorescence (89.6% at 645 nm) and THG (92.9% at 607 nm) signal wavelengths, making it efficient for both excitation and signal delivery. Heavy water (D_2O) immersion was used to avoid the excessive absorption of the commonly used immersion water, the latter of which degrades 3-photon signals by more than 20 times [9]. Using this objective lens, we acquired 3-photon fluorescence image of the crimson bead [Fig. 3(a)]. In biological sample of the mouse brain slice, THG image [Fig. 3(b)] excited at 1820-nm clearly reveals the myelinated axons in the white matter. These imaging results, based on transmittance characterization for the objective lenses, complement the wavelength coverage of MPM within the 1700-nm window.

4. Conclusion and Discussion

For optical microscopy, objective lens selection is one of the prerequisites in system building. In addition to other parameters such as NA, working distance, magnification, and immersion type, transmittance is no doubt a key parameter that needs to be known prior to objective lens selection, as virtually all imaging systems are targeting maximum excitation delivery to the sample and signal collection. Within the existent 800-nm, 1300-nm, 1700-nm, and the emergent 2200-nm NIR optical window, however, the transmittance data are not all available from either the literature or even the manufacturers themselves, especially at the 1700-nm and 2200-nm window. This poses a major obstacle for objective lens selection. Here combining the broadband detection capability of the spectrometer, and the wide-angle collection capability of the integrating sphere, we performed detailed transmittance measurement of eight objective lenses available to us, covering visible and all the four NIR optical windows. Based on these measured results, we also selected the objective lens with the highest transmittance performance for 1820-nm excitation, and demonstrated its application to both 3-photon fluorescence and THG imaging at this wavelength. We expect our results will provide guidelines for objective lens selection in initial system design, and will help if objective transmittance in existent systems is to be optimized.

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