Signal enhancement in multiphoton imaging by the use of coated glass substrates

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Abstract: In nonlinear optical imaging of biological specimens, more than half of the generated luminescence signal is lost, when signal collection is performed in the epi-illuminated geometry. In this study, we enhanced the collected luminescence signal by the use of alternating multiply-coated layers of tantalum pentoxide (Ta₂O₅) and silicon dioxide (SiO₂) on standard microscope cover glasses that has high transmission in the near-infrared wavelength region and high reflection of the visible, luminescence signal. Our coating is biocompatible, allows visual examination of the specimens and optimize collection of the luminescence signal. We demonstrated this approach on a number of specimens including sulforhodamine solution, fluorescence microspheres, and labeled 3T3 cells. In all cases, the use of coated cover glass enhanced signal, optimally by a factor of about 2. Image analysis of labeled 3T3 cells also shows signal enhancement did not contribute to additional photobleaching. Our results show that properly designed coated cover glass can enhance detected signal in multiphoton microscopy and result in improved image quality.

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1. Introduction

As in most forms of optical microscopic imaging, multiphoton microscopy relies on the use of glass substrates as the viewing window of biological specimens. With most microscope objectives corrected for the spherical aberration caused by the presence of a No. 1.5 thick cover glass, such substrate is ubiquitous in biological optical microscopy.

However, although the use of transparent glass substrates as cover slide or cover glass is convenient to researchers, the inherent loss of signal in the transmission (forward) direction is inevitable. Collection of specimen luminescence in the epi-illuminated direction is, at best, half of the total signal generated. Efforts to increase signal may lead to the use of higher excitation power which causes accelerated photobleaching. The invention and subsequent development of multiphoton microscopy have demonstrated that this technique is ideal for imaging three-dimensional tissue specimens [1,2]. While this imaging modality has found wide applications in thick specimens such as the brain and liver [3–5]. Other applications in multiphoton imaging of thinner specimens are significant. Such examples include drosophila embryo, engineered tissue specimens fluorescence correlation spectroscopy (FCS) and two-photon uncaging experiments [6–9]. In order to optimally enhance signal, different strategies have been developed to optimally enhance the collection of signal. Jonn White and associate proposed a second detection channel via the condenser to improve the collection efficiency of the specimen [10]. Enrico Gratton’s group through housing the microscope inside reflective containers optimized photon collection [11]. Previously, mirror slide composed of coatings that are 50 nm in gold or silver has been devised as a non-full-purpose solution [12]. In this work, we used a different approach. Our coating is designed for the optimization of reflected luminescence signal while allowing transmission of the near-infrared excitation source. Transmittance of excitation wavelength would reduce reflection back into the photomultiplier tubes for possible detection. The coating we use also allows standard white light examination of the specimens to be achieved.

The cover glass-based, coated system was designed and coated. Standard No. 1.5 cover glasses (22 × 22 mm² in size) were sent to Thin Film Technology Center at National Central University (Zhongli, Taiwan) where alternating layers of tantalum pentoxide (Ta₂O₅) and silicon oxide (SiO₂) were coated onto the cover glasses. In all, a multilayer structure composed of a total of 40 alternating layers of the two materials were coated onto the cover glasses. To optimize biocompatibility, the last layer in contact with the specimen is SiO₂. As the structure and spectrum of the coated cover glasses shows (Fig. 1), at 0 degrees incidence, the coatings are designed to transmit above 661 nm (50% transmission point). In the epiilluminated path, the coatings are designed to reflect below this wavelength. The transmission characteristics of the coated cover glasses conveniently allow standard white light examination of the specimens to be achieved.

2. Microscope systems and sample preparations

The multiphoton microscope system used for image acquisition is composed of a diode-pumped, solid-state laser system (Millennia eV, Spectra Physics, Mountain View, CA) which is used to pump a femtosecond, titanium-sapphire laser (Tsunami, Spectra Physics). 780 nm output of the laser was guided into a home-built microscope system. The system was incorporated with a commercial inverted microscope (TE2000U, Nikon, Japan). In all, three focusing objectives (S Fluor 10, NA 0.5; S Fluor 20x, NA 0.75; and Plan Apo 60x, NA 1.2 WI, Nikon) were used. The collected signals were spectrally resolved by the combination of dichroic mirrors (405dcxr, 530dcxr Chroma Technology) and filters (HQ465/70, HQ630/92).
for the respective detection in the blue and red channels. Each image area was scanned at 256 X 256 pixels resolution by using the 20x air immersion lens and the 60x water immersed objective. Note that for acquisition of white light and multiphoton imagine (Fig. 3), the specimen were moved to a similar microscope equipped with a camera for white light imaging. In this case, the 4 μm microspheres were imaged with a detection channel of 500-550 nm. All signals were detected by single-photon-counting photomultiplier tubes (R7400P, Hamamatsu, Hamamatsu City, Japan).

![Diagram of coated cover glass](image)

**Fig. 1.** Structure and spectrum of the coated cover glass. A total of 40 alternating layers of Ta2O5 and SiO2 were coated. For optimal biocompatibility, the layer in contact with the specimen is SiO2.

When scanning the uniform fluorescent solution of the sulforhodamine B sodium salt solution (1.5 mg/ml, Sigma), the 10x objective was used. The samples of uniform fluorescein solution of sulforhodamine B were prepared by sandwiching between two cover glasses spaced with electrical tape. In this manner, we were able to create thin-layer specimens with thickness close to the thickness of the cover glass and the bottom and top of the chamber were covered both standard cover glasses for the control group and normal and coated cover glasses for the experimental group. Another group of specimens we imaged were fluorescent microspheres (orange 1 μm F8820, green 4 μm F8859 and orange 10 μm F8833, Invitrogen). In preparing the fluorescent microsphere specimens, the beads were mixed with 70% ethanol and dried on the normal cover glass. The microsphere-contained cover glasses were covered with a normal cover glass for the control group and a coated cover glass for the experimental group. Next, the specimens were attached to the glass slide for multiphoton imaging. Finally, to verify the efficacy of coated cover glass on the biological specimens, 3T3 fibroblast cell line was labeled and imaged. However, when preparing the specimens with 3T3 cells, longer cover glasses were cultured with cells with 3 days after cell seeding. Before imaging, the cultured cover glasses were fixed with 10% formalin (Macron) and labelled the nucleus with Hoescht 33342 (Invitrogen) after 100x dilution (final labeling solution is 0.1 mg/ml in PBS). The cells were labeled for five minutes. Next, the cell- contained cover glasses were covered by the normal cover glass for the control group and the coated cover glass for the experimental group.
3. Results

Shown in Fig. 2 are the experimental setup (A) and integrated axial intensity profiles of 1.5 mg/ml sulforhodamine B solution sandwiched between two cover glasses. Note that regardless the use of 10x, 20x, or 60x objectives, one can observe the increase in signal enhance of sulforhodamine B fluorescence. The signal enhancement is most significant and comparable for 10x and 20x objectives and less significant for the 60x lens. Repeated trials displayed the similar tendency of profile in the individual case.

![Integrated axial intensity profiles](image)

Since the coated cover glasses allows partial transmittance in the visible range, one can visualize the microsphere distribution prior to multiphoton imaging (Fig. 3). Furthermore, we acquire the three-dimensional images of the fluorescent microspheres and determined the integrated axial intensity profiles with and without the use of coated cover glass. Shown in Fig. 4 is the integrated axial intensity profiles of 1 μm microsphere acquired with the 20x objective. After obtaining the axial intensity profile patterns, we determined the ratios of intensity enhancement for the 20x and 60x objectives for 1, 4, and 10 μm fluorescent microspheres. In all cases, the intensity of the fluorescent microspheres (10 per experiment) were enhanced with the use of coated cover glass with the enhancement ratio ranging from 1.22 to 1.99. Because fixed and small step-distance was used for all objective lens, a longer axial full width at half maximum (FWHM) was inevitable.

In the last set of experiments, we imaged the Hoechst 33342 labeled 3T3 cells for a simplified comparison. Images of the specimens with and without the use of coated cover glass are shown in Fig. 5. One immediately noticed that the use of coated cover glass significantly increased the intensity of fluorescence images. A better comparison could be done through a partial coated coverslip to demonstrate the difference of the same specimen. Subsequently, 5 nuclei of the labeled 3T3 cells were selected and their fluorescence determined as a function of time to determine the degree of photobleaching. As shown in Fig. 6, the initial signal of nuclei on coated cover glass was stronger in intensity by a factor of 1.92. Furthermore, the fluorescence dependence was fitted to an exponential function and it was found that photobleaching occurred at the same rate. Therefore, the use of coated cover
glass enhanced the signal by about a factor of two, while maintaining the same rate of photobleaching.

![Image](https://via.placeholder.com/150)

**Fig. 3.** White light and multiphoton excited fluorescence images of 4 μm microspheres with the use of coated cover glasses. 20x objective was used.

![Intensity profiles](https://via.placeholder.com/150)

**Fig. 4.** Intensity profiles on three different size of fluorescent bead and two objective lens were selected to compare the ratio of enhancement. (Top) Integrated axial intensity profiles of 1 μm microspheres acquired with the 20x objective. (Bottom) Ratios of the signal enhancement at different conditions were listed as a table.

| Depth (μm) | 1μm | 4μm | 10μm |
|-----------|-----|-----|------|
| 20x       | 1.99±0.91 | 1.27±0.31 | 1.43±0.42 |
| 60x       | 1.88±0.53 | 1.22±0.21 | 1.25±0.32 |
4. Conclusion

Through the use of a multiply-coated cover glass, signal enhancement of detected luminescence under multiphoton microscope imaging can be achieved. The high
transmittance of the coating in the near-infrared and reflectance in the visible allowed the enhancement of fluorescence signal collected in the epi-illuminated direction. Signal enhancement was found for uniform fluorescence solution, fluorescent microspheres, and labeled 3T3 cells. The current design is ideal for signal enhancement in multiphoton applications of thin biological specimens, without the additional introduction of specimen photobleaching.

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