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In Vivo Deep-Brain 3- and 4-Photon Fluorescence Imaging of Subcortical Structures Labeled by Quantum Dots Excited at the 2200 nm Window

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Abstract

Multiphoton microscopy (MPM) is an enabling technology for visualizing deep-brain structures at high spatial resolution *in vivo*. Within the low tissue absorption window, shifting to longer excitation wavelengths reduces tissue scattering and boosts penetration depth. Recently, the 2200 nm excitation window has emerged as the last and longest window suitable for deep-brain MPM. However, multiphoton fluorescence imaging at this window has not been demonstrated, due to the lack of characterization of multiphoton properties of fluorescent labels. Here we demonstrate technologies for measuring both the multiphoton excitation and emission properties of fluorescent labels at the 2200 nm window, using (1) 3-photon ($\eta\sigma_3$) and 4-photon action cross sections ($\eta\sigma_4$) and (2) 3-photon and 4-photon emission spectra both *ex vivo* and *in vivo* of quantum dots. Our results show that quantum dots have exceptionally large $\eta\sigma_3$ and $\eta\sigma_4$ for efficient generation of multiphoton fluorescence. Besides, the 3-photon and 4-photon emission spectra of quantum dots are essentially identical to those of one-photon emission, which change negligibly subject to the local environment of circulating blood. Based on these characterization results, we further demonstrate deep-brain vasculature imaging *in vivo*. Due to the superb multiphoton properties of quantum dots, 3-photon and 4-photon fluorescence imaging reaches a maximum brain imaging depth of 1060 and 940 μm below the surface of a mouse brain, respectively, which enables the imaging of subcortical structures. We thus fill the last gap in multiphoton fluorescence imaging in terms of wavelength selection.

Keywords: 2200 nm window; 3-photon fluorescence imaging; 4-photon fluorescence imaging; action cross section; multiphoton emission spectra.