Thesis Summary

Large-scale two-photon imaging of living mouse brain utilizing hydrophilized fluoropolymer nanosheet

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Introduction

Large-scale measurements in the living brains of animals have been used to reveal the anatomical and functional connectivity between brain regions and their underlying functions. *In vivo* two-photon microscopy visualizes neural activities and ensembles in living mammalian brains across multiple regions at a sub-cellular resolution.

To observe mouse brains by two-photon microscopy, the "open skull method" creates a cranial window in which a portion of the mouse skull is completely removed and replaced with a high transparent material such as a glass coverslip. However, bleeding at the brain surface is inevitable to remove the submillimeter-thick cranial skull, which results in deterioration of fluorescent signals due to the optical absorption of hemoglobin. In addition, the open skull method is usually employed for a small cranial window (2 - 4 mm in diameter) to avoid injury in large vessels and unnecessary bleeding. Generally, the size of cranial windows using a flat glass coverslip is restricted to a maximum diameter of 5 mm to avoid pressure on the brain tissue.

In this study, I propose a novel cranial window approach utilizing thin polymer film, also known as a nanosheet, as a sealing material for *in vivo* two-photon imaging with a broad field of view (FOV). Nanosheets at approximately 10–100-nm thickness has unique properties, including high adhesion strength, flexibility, and transparency. To apply a nanosheet to a living mouse brain, I utilized a polyethylene-oxide coated CYTOP (PEO-CYTOP) nanosheet with a thickness of ~130 nm, in which a single-side surface was hydrophilized by polyethylene oxide (PEO). These PEO-CYTOP nanosheets firmly adhered to brain surfaces, which suppressed bleeding from superficial veins. Furthermore, I proposed the NIRE (Nanosheet Improved by light curable resin) method to make a

cranial window that used both the PEO-CYTOP nanosheet and UV curable resin. The NIRE method is aimed at *in vivo* multi-scale, long-term imaging of neural structures and neural activities in mouse brains.

Materials and Methods

Animals

I used adult mice (over 8 weeks of age, regardless of gender) for all experiments. All mice were housed under a 12 h/12 h light/dark cycle. I used wild-type mice of the C57BL/6J strain for expression of Ca^{2+} indicator by an adeno-associated virus (AAV). I used Thy1-EYFP-H (H-line) transgenic mice for *in vivo* imaging in this study. H-line mice, which express the enhanced yellow fluorescent protein (EYFP) in a subset of layer 5 pyramidal cells, enabled to achieve fluorescence images that were suitable for the visualization of the structure of neurons. GLT1-G-CaMP7 (G7NG817) transgenic mice expressing the Ca^{2+} indicator G-CaMP7 in mainly astrocytes and a subpopulation of neurons were used to evaluate Ca^{2+} activity.

Cranial window surgery

The mouse was anesthetized with 1% isoflurane during surgery. The body temperature of the mouse was maintained using a heating light and/or mat. First, the hair and skin of the mouse were removed to expose the cranial skull. Subsequently, part of the cranial skull was removed by a dental drill to generate a cranial window for *in vivo* imaging of the living mouse brain at high resolution.

After washing dust and blood with phosphate-buffered saline (PBS) or saline, I placed the PEO-CYTOP nanosheet supported by a nonwoven fabric onto the brain surface and attached the hydrophilic side to the brain surface. The PEO-CYTOP nanosheet was then lightly pushed via the nonwoven fabric using a tweezer, for adherence to the brain surface. After confirming that the PEO-CYTOP nanosheet had been transferred to the brain

surface, the nonwoven fabric was removed. If the misplacement of the nanosheet occurred, I was able to easily remove it by dropping PBS or saline onto it and floating the nanosheet from the brain surface. After attaching it, the nanosheet was adjusted to the specified range of the cranial window. The edge of the sheet was glued using cyanoacrylate or blue light curable resin, to prevent soaking of the immersion solution under the PEO-CYTOP nanosheet. When fixing the PEO-CYTOP nanosheet, the resins remained localized at the edge of the nanosheet because CYTOP exhibits chemical resistance and repellency to both water and oil. However, in the case of observations performed using an air-immersion objective lens, the PEO-CYTOP nanosheet and its edge did not require sealing with the resins.

Image acquisition

All two-photon fluorescence images were obtained by two-photon laser microscopy customized for *in vivo* imaging (A1R-MP+, Nikon). I used Olympus XLFLUOR4X/340 4×/0.28 NA air-immersion objective lens for *in vivo* imaging with a broad FOV, Nikon CFI75 LWD 16×/0.80 NA water-immersion objective lens for broad cross-sectional imaging, and a Nikon Apo LWD 25×/1.10 NA water-immersion objective lens for deep *in vivo* imaging.

To measure wide-field Ca^{2+} elevations, I used epi-fluorescence microscopy (AZ100, Nikon) with a Nikon AZ-Plan Fluor 2×/0.20 NA air-immersion objective lens and a mercury lamp (Intensilight C-HGFI, Nikon) as the excitation light source.

Result

As a sealing material for cranial windows in the open skull method, PEO-CYTOP nanosheets were confirmed to firmly adhere to brain surfaces resulting in the suppression of bleeding from superficial veins. Next, using the flexibility of PEO-CYTOP nanosheets, I successfully made a large cranial window approximately the size of the whole parietal region. The window allowed for *in vivo* two-photon imaging of neural structure with a broad FOV at a high spatial resolution on the parietal region of Thy1-EYFP-H transgenic mice. To demonstrate wide-field imaging on the whole parietal region, I used Ca^{2+} imaging by epi-fluorescent microscopy in the G7NG817 transgenic mouse expressing a Ca^{2+} indicator G-CaMP7 primarily in astrocytes. To verify long-term availability, a small cranial window with a PEO-CYTOP nanosheet was covered with a glass coverslip to protect the brain surface against foreign substances and injury. Up to 9 weeks after the initial surgery, the transparency of the cranial window was maintained. Importantly, *in vivo* two-photon Ca^{2+} imaging at a single-cell resolution was achieved through the cranial window of G7NG817 transgenic mice at this time point.

Furthermore, I improved the cranial window method by NIRE method. This method was developed to suppress motion artifacts in awake mice and to enable long-term imaging of the living mouse brain with a broad FOV. Utilizing the NIRE method, I successfully visualized various neural structures in multi-scale ranging from the whole parietal region to single dendritic spines of a neuron in the Thy1-EYFP-H transgenic mouse. Furthermore, the NIRE method facilitated *in vivo* multi-scale Ca^{2+} imaging for neural activities from a population of several hundred neurons to single dendritic spines in jGCaMP7 expressing mouse brains. Notably, the cranial window maintained

transparency for over 166 days, likely due to the suppression of inflammatory reactions. Finally, the NIRE method successfully demonstrated *in vivo* 3D imaging of Thy1-EYFP-H transgenic mice through such a large cranial window from the cerebral cortex to the cerebellum.

Discussion

The PEO-CYTOP nanosheet sealed the injured blood vessels tightly and suppressed bleeding from the brain surface because of the high adhesive strength resulting from physical adsorption, such as van der Waals interactions. Previously, several biocompatible nanosheets, including a poly(L-lactic acid) (PLLA) nanosheet, were applied for the suppression of bleeding from various organs, including the stomach, liver, and inferior vena cava. In contrast, CYTOP nanosheets exhibited the potential as a material for living-tissue imaging because of their excellent water retention and surface adhesion. However, CYTOP nanosheets could not be attached firmly to the brain surface because of the high hydrophobicity of CYTOP. To overcome this, the adhesive surface of the CYTOP nanosheet was modified by coating it with PDMS and attaching PEO. This hydrophilization of the nanosheet surface enhanced the adhesion strength to wet specimens, resulting in the suppression of bleeding from the brain surface. Thus, the PEO-CYTOP nanosheet could apply to effectively prevent bleeding from the wet surface of living tissues other than the brain.

The optical disturbance caused by the PEO-CYTOP nanosheet was almost negligible compared with the conventional glass coverslip because the thickness of the PEO-CYTOP nanosheet was ~10^{-3} smaller than that of the glass coverslip (PEO-CYTOP nanosheet, ~130 nm; conventional glass coverslip, ~170 µm), and even smaller than the wavelength of visible light. Moreover, the refractive index of CYTOP is ~1.34, which is most like that of water (~1.33) but lower than that of glass (~1.52). Furthermore, the PEO-CYTOP nanosheet had approximately 100% transmittance. Therefore, the PEO-CYTOP nanosheet can be used as a sealing material for *in vivo* brain imaging with minimal effects

from incident lights.

In conclusion, a PEO-CYTOP nanosheet as a sealing material for a cranial window in the open skull method successfully enabled the visualization of neural morphology and Ca^{2+} elevations with a broader FOV in the mouse brain. Furthermore, the NIRE method allowed *in vivo* multi-scale imaging of neuronal structures and Ca^{2+} activity ranging from a spine of a single neuron to several hundred neurons in the same mouse brain. The NIRE method also facilitated a super large cranial window covering the cerebral cortex to the cerebellum. This novel technique may promote the understanding of the anatomical and functional connectivity between multiple cortical regions in living animal brains by improving the surgical procedure and expanding the optically observable regions.