博士（理学）　張　　郁芬

学位論文題名

Development of technologies for imaging and manipulation of Ca^{2+} dynamics in living cells

（生きた細胞内における Ca^{2+} 動態の可視化と操作を行う技術の開発）

学位論文内容の要旨

Ca^{2+} is an important second messenger that controls synaptic transmission and hormonal release in living cells. Several synthetic or genetically encoded Ca^{2+} indicators (GECIs) based on fluorescence have been developed, allowing us to perform Ca^{2+} imaging in living cells with fine spatiotemporal resolution. Very recently, optogenetic tools such as channelrhodopsin2 (ChR2) have enabled to operate behavior of whole organisms by light-mediated manipulation of neuronal activity. To evaluate neuronal activity during optogenetic implementation, fluorescent indicators have been utilized. However, the action spectrum of ChR2 extends from the ultraviolet through to ~550 nm, severely restricting the ability of researchers to image neuronal activation with current popular genetically encoded or synthetic dye-based indicators.

In addition, the issues of phototoxicity, photobleaching, and specimen auto-fluorescence have to be considered when choosing a fluorescent protein. Thus, bioluminescent proteins such as luciferase are superior for observing biological processes since their signals can be acquired without an external light source. Alternatively, the red-shifted Ca^{2+} indicators offer the advantages of a diminished auto-fluorescent background with longer wavelength light. Here, I present a newly developed bioluminescent indicator and demonstrate a novel system using red-shifted fluorescent indicators for compatible fluorescence imaging with optogenetic activation.

In **Chapter 1**, the low intensity of bioluminescence was improved by increasing brightness

through conducting random mutagenesis of RLuc8. I identified a brighter mutant, RLuc8_S257G, and then applied the mutant in a bioluminescence resonance energy transfer (BRET) system by fusion with a yellow fluorescent protein to greatly increase the light output. Finally, the fusion protein presented approximately 8-fold higher peak intensity than RLuc8 alone and was named VRL10.3_S257G.

In **Chapter 2**, I took advantage of the higher intensity of BRET-based indicators and the larger signal change of complementation of split luciferase (CSL)-type indicators (in which the luciferase molecule emits the light through the proper reconstitution of N-terminus and C-terminus of luciferase) by designing a CSL and BRET hybrid Ca^{2+} indicator. The calcium sensing domain (CaM-M13) was inserted into the RLuc8 moiety of VRL10.3_S257G, named SuperBRAC, which displayed a high dynamic range, high S/N ratio and fine spatial resolution. The split point 228^{th} of the RLuc8 moiety presented a higher intensity and dynamic range compared with the others. Histamine-induced Ca^{2+} oscillation in HeLa cells indicated that the complementation of the split N-terminus and C-terminus of luciferase was reversible. The first bioluminescence imaging taken in video-rate at the single cell level was achieved by this indicator, which is essential for detecting rapid kinetic motion such as neuronal responses.

In **Chapter 3**, I demonstrated the performance of various Ca^{2+} indicators for recording neuronal activity and photo-activation of ChR2 with Ca^{2+} imaging in dissociated neurons. Furthermore, a red-shifted Ca^{2+} indicator, providing deeper tissue penetration associated with imaging with longer wavelength light, for conjunction with ChR2 activation was examined in brain slices.

In **Chapter 4**, I constructed a simple optic system for compatible operation of an optogenetic tool with fluorescence imaging, with which I could investigate rapid Ca^{2+} dynamics in neuron cells in real-time. I revealed the biphasic property of the Ca^{2+} increase in response to optical stimulation.

These achievements open the door to a new era for researchers across wide fields.

学位論文審査の要旨

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博士学位論文審査等の結果について（報告）

近年、光遺伝学的技術により細胞や細胞内生体分子の機能を光照射依存的に制御し、細胞内で惹起される現象を蛍光イメージングにより分子レベルで解析する研究が盛んになりつつある。従来、光遺伝学的操作と蛍光画像取得はシーケンシャルに行うのが一般的であったが、この場合蛍光画像を撮影している間に光遺伝学操作は行うことができず、またその逆も然りであった。本論文は蛍光イメージングで汎用されている CCD カメラの画像取得フレーム間に存在する従来見過ごされてきた“画像転送時間および電荷消去時間”いわゆる「デッドタイム」に着目し、CCD カメラからのタイミングシグナルをファンクションジェネレーターに導入し、任意の周波数と波形を持った交流電圧信号に変換後、LED 光源へトリガーシグナルとして導入することで、蛍光イメージングの時間分解能を犠牲にすることなく、光遺伝学的操作を行うことが可能なシステムを開発した。著者は、本システムを用いて神経細胞の光照射依存的な興奮を新規に開発した赤色蛍光 Ca^{2+}指示薬により高速可視化することに成功し、Ca^{2+}の 2 段階応答性と光刺激量との関係を明らかにした。また、原理的に励起光照射を必要としない化学発光タンパク質性 Ca^{2+}指示薬も開発し、光遺伝学的ツールによる多機能制御の可能性を示した。この業績は光遺伝学研究の新しい展開に寄与するところ大なるものがある。

よって著者は、北海道大学博士（理学）の学位を授与される資格あるものと認める。