様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Anisotropic magnetic field effect imaging microspectroscopy: a technique to search cells for magnetic compass ability

研究課題名（英文）Anisotropic magnetic field effect imaging microspectroscopy: a technique to search cells for magnetic compass ability

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研究成果の概要（和文）：本研究では、光化学反応の異方性磁場効果を微小サンプルで観測する新しい顕微鏡の開発を行った。動物の磁気コンパス能力と磁場が人間の健康に影響を及ぼす可能性に焦点を当て、磁場が生物学的プロセスに影響を与える様式の知見を提供するために、本研究対象を細胞レベルでの生物系とした。
完成した顕微鏡は、1）細胞内自家蛍光の磁場効果を世界初の直接観測、2）高輝度蛍光色素を用いた拘束系でのラジカル対システムの開発、に適用された。 これらは、理論予測と比較した異方性磁場効果の詳細なモデル解明を可能にし、さらに単一ラジカル対の電子スピン効果の調査、磁場応答を利用して局所環境を可視化する生体センサーの開発を可能にする。

研究成果の学術的意義や社会的意義
人間を含む全ての動物が、自然環境に存在する弱い磁場の影響を受ける可能性を理解することは、人類にとって重要な問題である。それは、人間と動物の感覚に関する根本的な疑問と、弱い磁場が健康に及ぼす潜在的な影響に直接関係するからである。
本研究では、リアルタイムイメージングにより、磁場が生きているネイティブな状態の細胞に影響を及ぼしているという最初の直接的な証拠を提供している。これは、本研究分野において総論的な理解へ一歩前進させる重要な実験的観測を意味する。
本報告書では、当該分野の研究者に向けて、生体系における磁場感受性光化学プロセスの検出と応用方法の新し

研究成果の概要（英文）：This project involved the development of a new microscope for observing anisotropic effects of magnetic fields on photochemical reactions in microscopic samples. The target is biological systems (at the cellular level) to provide insights into the way magnetic fields can influence biological processes (with a focus on both the magnetic compass ability of animals and the possible effects of magnetic fields on human health).
The completed microscope was applied in two main areas: 1) the first direct observation of magnetic field effects on native cellular autofluorescence. 2) Development of constrained radical pair systems built from fluorophores of high brightness. These allow for detailed model investigations into anisotropic magnetic field responses for comparison with theoretical predictions and enable investigations of electron spin effects in single radical pairs and the development of biological sensors that exploit magnetic field responses to report on local environments.

研究分野：生物物理学

キーワード：磁気受容　ラジカル反応　磁場効果　蛍光顕微鏡　スピン化学

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様　式　C－１９、F－１９－１、Z－１９（共通）

1. 研究開始当初の背景

An important unsolved problem in behavioral biology is to understand the mechanism by which many animals are capable of sensing and responding to the Earth's magnetic field, which is extremely weak (approximately 30-50 μT). Both magnetic compass and magnetic map abilities are observed in the animal kingdom and based primarily on the observed effects of visible and applied radiofrequency radiation on the magnetic sensing ability of migratory birds, the favored mechanism for magnetic compass behavior involves the photochemical generation of a radical pair and its subsequent spin-selective reaction. Migratory birds demonstrate an inclination based magnetic compass ability which requires that the underlying magnetic reception mechanism must demonstrate sensitivity to both the magnitude and direction of an external MF. However, this hypothesis is not yet convincingly proven, despite a large number of studies over a long period of time. One key problem is that the ability to sense the magnitude and direction of the earth's magnetic field with sufficient sensitivity has not been observed at the cellular level.

2. 研究の目的

The goal of this research was to tackle the extremely challenging problem of providing robust experimental evidence for magnetic compass ability at the cellular level. Therefore, the aim of this research was to achieve the two followings:

(i) to develop a microscope capable of observing anisotropic magnetic field effects on spin correlated radical pairs (RPs).

(ii) to provide the first evidence for the anisotropic magnetic field effects at the cellular level.

3. 研究の方法

This work involved the development of a new fluorescence microscope to directly investigate photochemical reactions proceeding through the formation of radical pairs at the cellular level. To do so, the instrument must possess the following capabilities:

(i) The ability to apply a magnetic field along any arbitrary direction relative to the sample.

(ii) The ability to detect small (a few percent) changes in radical pair reactions at cell-level concentrations in cell level volumes

Moreover, this work involved the establishment of single molecule spectroscopy in order to meet the sensitivity requirements and to obtain some insights into the maximum sensitivity of RPs, which can be hidden by ensemble averaging, and the unique magnetic anisotropy of individual RP spin states.

4. 研究成果

1) *Anisotropic magnetic field effect fluorescence microscopy*

During the project period, the microscope capable of investigating anisotropic magnetic field effects on cellular samples, was successfully constructed, optimized and tested. The construction involved the installation of a projected vector field electromagnet GMW 5204 (the first commercial unit worldwide) to supply the field directly to the microscopic sample. This unique magnet is capable of generating a magnetic field along any arbitrary direction and this field can be rotated along any axis at audio frequencies.

To demonstrate the ability of the microscope, **Figure 1** shows fluorescence decays of three flavin based solution samples under magnetic fields applied along 3 perpendicular directions (25 mT amplitude and 1.0 Hz sine modulation). **Figure 1A and 1B** are the fluorescence decays of 10 μM flavin adenine dinucleotide (FAD) + 300 μM tryptophan (TRP) and 10 μM FAD in an acidic solution, respectively. These samples serve as positive control experiments and show that the microscope can detect the magnetic field effects of a few percent from typical flavin based radical pairs. **Figure 1C** presents the fluorescence decays of 10 μM flavin mononucleotide (FMN) in an acidic solution sample. This sample serves as a negative control experiment (because FMN does not produce radical pairs in the absence of a suitable electron donor molecule), and demonstrates that the microscope has no artefactual response to the magnetic field applied along any of the 3 perpendicular directions. The sample thickness was 2.0 – 2.9 microns, the flavin concentration was equal to typical endogenous flavin concentrations in animal cells, and the detection volume was smaller than the volume of typical cultured cells.

Therefore, the microscope developed during the project period demonstrates the capabilities and sensitivity necessary to enable the search for anisotropic magnetic field sensitivity in cells.

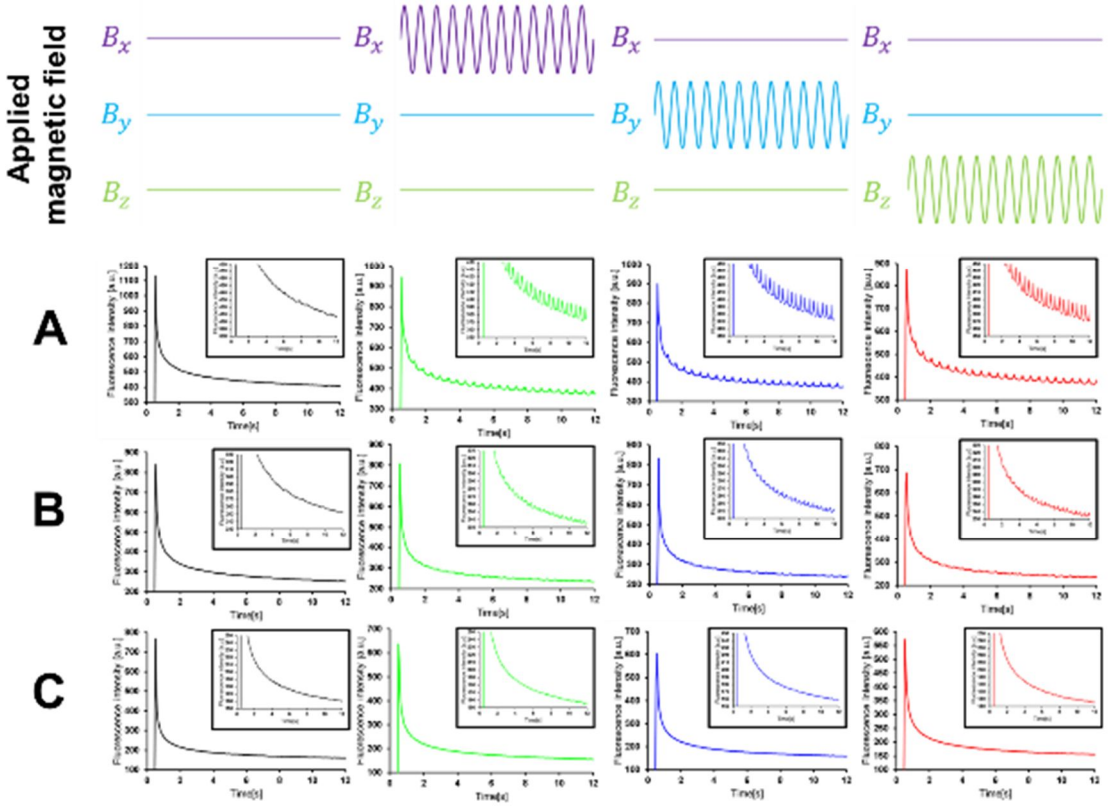

Figure 1. The fluorescence decays of flavin based solution samples under magnetic fields applied along 3 perpendicular directions (25 mT amplitude and 1.0 Hz sine modulation). (A) 10 µM FAD + 300 µM TRP in pH 2.3 buffer solution. (B) 10 µM FAD in pH 2.3 buffer solution. (C) 10 µM FMN in pH 2.3 buffer solution.

2) *Direct detection of magnetic field effects on native cell autofluorescence*

During the project period, we successfully established cell culture experiments in our laboratory and ultimately provided the first ever observation of direct magnetic field effects on native HeLa cell autofluorescence using the microscope described above.

A typical magnetic field effect on native HeLa cells is shown in **Figure 2**. **Figure 2A** shows merged images composed of bright field images captured before irradiation and fluorescence images captured after irradiation, where the blue circle indicates the position of the irradiation region. **Figure 2B** shows the change of average fluorescence intensity in the region of interest under the influence of a modulated magnetic field. The fluorescence decay curves show a small, but clear fluorescence change corresponding to the frequency of the applied modulated magnetic field sweep. In order to more clearly examine this change in the average fluorescence intensity, curves were fitted to the data from **Figure 2B** using the least squares method, and the residuals (observed value – fitted curve) were normalized using the fitted curve, resulting in a measurement of the fractional MFE (**Figure 2C**). In addition, our findings were rationalised in terms of magnetic field effects on photoinduced electron transfer reactions to flavins, through the radical pair mechanism. At the time of preparing this report, this work has been submitted for publication and is currently under review.

This breakthrough result not only demonstrates the ability of our instrument to observe, within sub-cellular assemblies, small magnetically induced changes in photochemistry at very low concentrations (cellular level flavin concentrations are typically of the order of a few micromolar), but also opens up a new vista for considering cellular level responses to magnetic fields.

As a future perspective, we will investigate the magnetic field effect on autofluorescence of a range of different cultured animal cells with the aim to ultimately make measurements in tissue samples. In addition, our current studies now focus on the observation of anisotropic field responses. Furthermore, we can use our new approach to critically test candidate molecules that may play a role as biological magnetoreceptors directly in living cells, for example the flavin based flavin based photoreceptor proteins proposed as responsible for the avian compass – the cryptochromes.

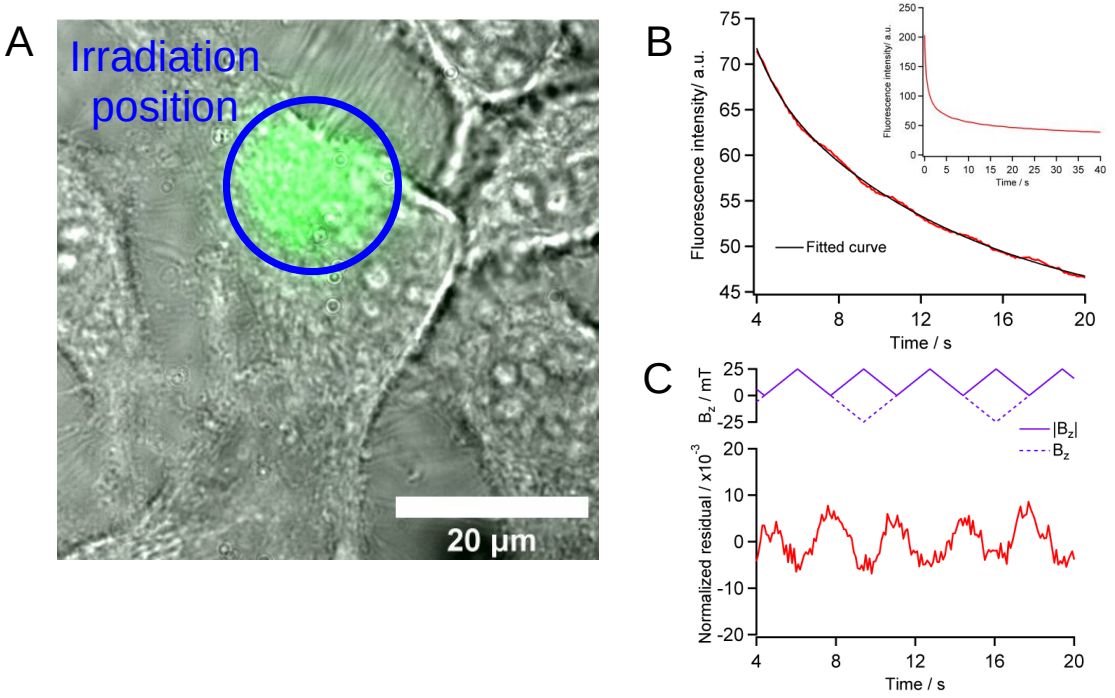

Figure 2. Magnetic field effect on the autofluorescence of HeLa cells. (A) Merged bright field and fluorescence images of a representative HeLa cell showing a magnetic field response. (B) Averaged autofluorescence change of the irradiated region of the HeLa cell with the application of a modulated external magnetic field (triangle wave, 0.15 Hz of frequency, 25 mT of amplitude). The insert figure shows the complete time period of the experiment. (C) Normalized residual intensity calculated by the average autofluorescence intensity divided by the value of the fitted curve representing the fractional MFE.

3) *Establishment of experimental systems for the observation of anisotropic magnetic field effects*

To study anisotropic magnetic field effects using the developed microscope, we have developed new experimental systems. In particular, during the project period, the establishment of single molecule spectroscopy of radical pairs has been carried out.

First, by employing a realistic kinetic scheme of FAD photochemistry based on published rate coefficients, we investigated the possibility of observing magnetic field responses on single flavin radical pairs. FAD was chosen, as it is the chromophore of the candidate magnetic field sensor proteins, the cryptochromes. We proposed a full experimental detection scenario to explore spin effects on the fluorescence of single FAD molecules. This work was published in Molecular Physics (international, peer reviewed journal). However, we concluded that the observation of magnetic field effects on single FAD molecules is feasible but experimentally challenging for measurements on integrated fluorescence intensity and fluorescence event statistics. The main reason is the weak fluorescence emission of the fluorophore.

Based on this work we developed strategies for our experimental approach and progressed to develop magnetic sensitive photoreaction systems with very high fluorescence brightness during the project period. The development was achieved using a number of different single molecule fluorophores and electron donor molecules.These systems show magnetic field responses of similar size to the flavin based radical pairs, but have fluorescence imaging brightness up to two orders of magnitude greater. In addition, we tested a wide range of approaches for immobilising the radical pairs (in thin gel and polymer films) but finally settled on establishing an immobilization system for single molecule measurements which works by conjugating the single molecule fluorophores with proteins and attaching the proteins to the glass surface of the sample slides. (**Figure 3**).

Currently, the immobilization system and the microscope sensitivity for single molecule measurements is being optimized, and both experiments are now underway to record both ensemble and single-molecule measurements of the anisotropic magnetic field response. Moreover, the bright systems can be applied not only to measuring electron spin effects on single radical pairs, but also to bioimaging using magnetic field effects as a measure of radical pair environment, and such work is expected to seed new approaches in interdisciplinary research across spin chemistry

and molecular biology. Preparations for such experiments are also currently being undertaken.

As an unexpected result, a photopolymerization process between excited FMN and hen egg white lysozyme (HEWL) was discovered and subsequently similar related processes in tryptophan based radicals were also revealed. After continuous excitation for around three hours, sufficient polymerisation occurs for the solid substance to be observed visually in the microscope images which capture the irradiation area (**Figure 4**).

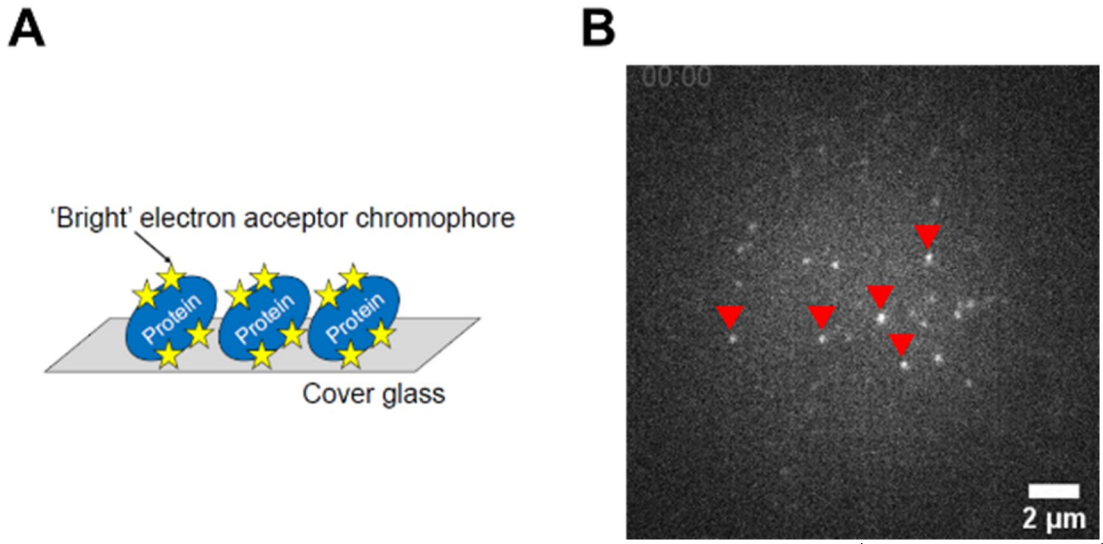

Figure 3. Single molecule proteins conjugated with bright fluorophores (protein: dye ≈ 1: 4) adsorbed on glass surface. (A) Schematic of protein adsorption (B) Fluorescence image of single molecule proteins adsorbed on cover glass. The red arrows show fluorescence signal from single molecule proteins.

The fluorescence image in **Figure 4A** shows the fluorescence of FMN, the brightfield image in **Figure 4B** shows the formation of the solid, and the merged image in **Figure 4C** shows the strong fluorescence arising from the accumulation of FMN in the solid. Since this polymerization does not occur only with FMN, it is concluded that HEWL formed the solid substance. This suggests that FMN was trapped in the solid polymer formed from the HEWL proteins.

The magnetic field effects on this reaction evolve as the polymer grows, providing direct insight into the radical pair environment. The full mechanism describing the process is still under investigation and will be published when the results and analyses are completely conclusive. It is worth noting that as this system also showed a magnetic field effect it may be possible to use it as a means of immobilizing flavin based radical pairs to help provide insight into the mechanism of animal magnetoreception based on cryptochromes which employ such flavin based radical pairs. This system is more closely related to the anisotropic magnetic field effect of the animal magnetic compass than the bright magnetic sensitive photoreaction system. Currently, the anisotropic magnetic field response in this system is also under investigation using the developed microscope.

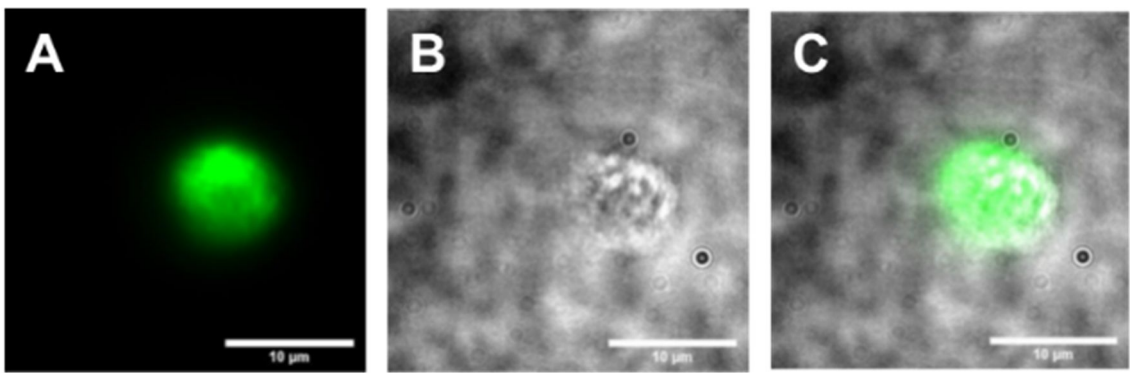

Figure 4. A polymeric substance formed between FMN and HEWL after continuous irradiation for around three hours. (A) The fluorescence image. (B) The bright field image. (C) The merged fluorescence and bright field images.

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　0件）

１．著者名 Noboru Ikeya, Egor A. Nasibulov, Konstantin L. Ivanov, Kiminori Maeda & Jonathan R. Woodward	４．巻 Online
２．論文標題 Single-molecule spectroscopy of radical pairs, a theoretical treatment and experimental considerations	５．発行年 2018年
３．雑誌名 Molecular Physics	６．最初と最後の頁 Online
掲載論文のDOI（デジタルオブジェクト識別子） 10.1080/00268976.2018.1559954	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計18件（うち招待講演　7件／うち国際学会　5件）

１．発表者名 Jonathan Woodward
２．発表標題 Microspectroscopy of flavin-based radical pairs
３．学会等名 Spin Chemistry meeting（招待講演）（国際学会）
４．発表年 2019年

１．発表者名 Noboru Ikeya
２．発表標題 Magnetic field effect fluorescence microscopy for in vivo and anisotropic field measurements on flavin based radical pairs
３．学会等名 Spin Chemistry meeting(（国際学会）
４．発表年 2019年

１．発表者名 Jonathan Woodward
２．発表標題 Microscopic studies of spin effects in flavin-based radical pairs
３．学会等名 Annual Meeting on Photochemistry 2019
４．発表年 2019年

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Noboru Ikeya

２．発表標題
Magnetic field effect fluorescence microscopy for in vivo and anisotropic field measurements on flavin based radical pairs

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２．発表標題
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The Society of Electron Spin Science and Technology (SEST) Annual Meeting - Joint NMR / EPR Symposium（招待講演）

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３．学会等名
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３．学会等名
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３．学会等名
Annual Meeting on Photochemistry 2018

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2018年

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Time resolved microscopy of spin-sensitive radical pair reactions

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２．発表標題
Fluorescence microscopy based detection of spin effects on single radical pairs’

３．学会等名
Annual Meeting of the Society of Electron Spin Science and Technology

４．発表年
2018年

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２．発表標題
Microspectroscopy of flavin based radical pairs

３．学会等名
Annual Meeting of the Society of Electron Spin Science and Technology

４．発表年
2018年

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３．学会等名
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３．学会等名
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４．発表年
2017年

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２．発表標題
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３．学会等名
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４．発表年
2017年

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４．発表年
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１．発表者名
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２．発表標題
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３．学会等名
3rd Kanto Area Spin Chemistry (KASC) Meeting（招待講演）

４．発表年
2017年

〔図書〕　計0件

〔産業財産権〕

〔その他〕
Research resulting from this work entitled "Cellular autofluorescence is magnetic field sensitive" is currently under review by the journal, Proceedings of the National Academy of Sciences (PNAS)

６．研究組織

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