Chemical Physics Letters 630 (2015) 32–36

Contents lists available at ScienceDirect

Chemical Physics Letters

journal homepage: www.elsevier.com/locate/cplett

# Solvent dependence of two-photon absorption spectra of the enhanced green fluorescent protein (eGFP) chromophore

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ARTICLE INFO

*Article history:*
Received 2 March 2015
In final form 21 April 2015
Available online 30 April 2015

ABSTRACT

Two-photon absorption spectra of 4′-hydroxybenzylidene-2,3-dimethylimidazolinone, a model chromophore of enhanced green fluorescent protein (eGFP), were measured in various solvents. The two-photon absorption band of its anionic form is markedly blue-shifted from the corresponding one-photon absorption band in all solvents. Moreover, the magnitude of the blue shift varies largely depending on the solvent, which does not accord with the assignment of the two-photon absorption band to the transitions to the vibrationally excited $S_1$ state. Our finding is readily rationalized by considering overlapping contributions of the $S_1 \leftarrow S_0$ and $S_2 \leftarrow S_0$ transitions, suggesting the involvement of the $S_2$ state also in two-photon fluorescence of eGFP.



## 1. Introduction

Since the discovery of the green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria*, various fluorescent proteins (FPs) have been developed as fluorescent markers and have been extensively used in the fields of cell and molecular biology [1–3]. Among the imaging techniques utilizing FPs, two-photon excitation fluorescence microscopy has substantial advantages over one-photon excitation microscopy, such as deep penetration depth, reduced background fluorescence, and reduced photodamage [4,5]. High-resolution microscopy of intact tissues has been achieved with the two-photon excitation scheme, enabling visualization of deep tissues such as lymphatic organs, kidney, heart, skin and brain [6,7]. In spite of these wide applications, fundamental issues regarding the fluorescence mechanism with two-photon excitation of FPs have been left unresolved [8–13]. So far, two-photon fluorescence excitation spectra of several FPs have been examined to obtain insights into the fluorescence mechanism [5,9,10,14]. Interestingly, the two-photon fluorescence excitation spectrum of enhanced green fluorescent protein (eGFP, S65T/F64L) appears blue-shifted compared with the one-photon fluorescence excitation spectrum [5,9,10]. This discrepancy is unexpected given that all relevant electronic transitions should have nonzero intensity both in one-photon absorption (OPA) and two-photon absorption (TPA), because the chromophore lacks inversion symmetry (Figure 1a, right). Blab et al. attributed the blue shift to the transition from the $S_0$ state to the vibrationally excited $S_1$ state (Figure 2a) [9]. However, the reported two-photon fluorescence excitation spectra only had a limited number of data points, which made quantitative discussion difficult. It was highly desirable to obtain high-quality two-photon absorption spectra to further elucidate the mechanism of the two-photon fluorescence of FPs.

Previously, we reported precise TPA spectra of eGFP using the nondegenerate multiplex method [15]. We observed that the TPA band is blue-shifted compared with the OPA band, and the magnitude of the blue shift was determined as 1050 $cm^{-1}$. In addition, we also measured the TPA spectrum of a model chromophore of eGFP, 4′-hydroxybenzylidene-2,3-dimethyl imidazolinone (HBDI, Figure 1b), in methanol, and showed that the anionic form of HBDI exhibits a similar blue shift. These results indicate that the difference between the OPA and TPA spectra arises from the intrinsic electronic structure of the chromophore itself. Interestingly, the blue shift of 670 $cm^{-1}$ for HBDI is markedly different from that of 1050 $cm^{-1}$ for eGFP. If the blue shift of TPA is due to the transition from the $S_0$ state to the vibrationally excited $S_1$ state as claimed [9], the difference in the blue shift (1050 $cm^{-1}$ vs. 670 $cm^{-1}$) should correspond to the difference in the vibrational energy. However, it is very unlikely that the vibrational energy varies so greatly with the change in the environment (i.e., in protein and in solution). Therefore, the strong environment dependence of the magnitude of the blue shift suggests that the assignment of the blue shift to

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http://dx.doi.org/10.1016/j.cplett.2015.04.028
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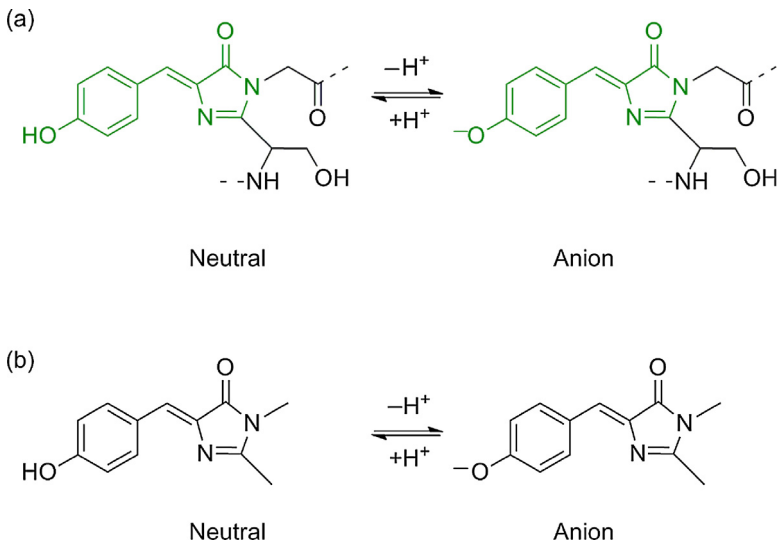


**Figure 1.** Chemical structures and the acid–base equilibrium for (a) the chromophore of eGFP and (b) HBDI.

the vibrational energy is inappropriate. To explain our observation, we proposed the involvement of another electronic excited state, the $S_2$ state, which has larger two-photon absorptivity and smaller one-photon absorptivity than the $S_1$ state (Figure 2b) [15]. In this mechanism, the TPA band consists of the smaller $S_1 \leftarrow S_0$ and the larger $S_2 \leftarrow S_0$ contributions, making it appear blue-shifted compared with the OPA band. The energy difference between the peak maxima of the OPA and TPA bands reflects the energy difference between the $S_1$ and $S_2$ states.

After our study, high-quality two-photon fluorescence excitation spectra of eGFP were measured by several groups, and the similar blue shift was reported [12,13,16]. However, Drobizhev et al. associated the blue shift with the transition to the vibrationally excited $S_1$ state and did not consider the involvement of the $S_2$ state [12,13,17]. Thus, at the moment, two different mechanisms have been proposed for the same observation. To consider which mechanism is more plausible, the solvent dependence of the OPA and TPA spectra of HBDI is very informative. In the case that the TPA arises from the vibrationally excited $S_1 \leftarrow S_0$ transition, the magnitude of the blue shift should be almost the same even if the solvent is changed, because the solvent dependence of the vibrational energy is very small in general.

In this work, we measured the TPA spectra of HBDI in various organic solvents, focusing on the energy difference between the OPA and TPA bands. On the basis of the obtained results, we discuss the possibility of the transition to the vibrationally excited $S_1$ state and the plausibility of the involvement of the $S_2$ state in TPA of the anionic form of HBDI.

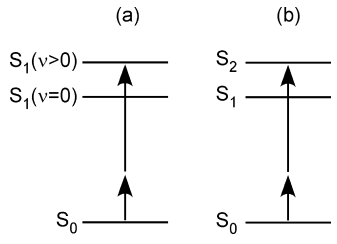


**Figure 2.** Two different mechanisms for the blue shift observed in the TPA spectra of the anionic form of HBDI and eGFP. (a) Transition to the vibrationally excited $S_1$ state. (b) Transition to another electronic excited state. The $S_2$ state exists in the vicinity of the $S_1$ state, and exhibits a larger two-photon absorptivity and smaller one-photon absorptivity.

## 2. Experimental

The experimental setup for the TPA measurements has been described elsewhere [15]. Briefly, a narrow-band laser pulse of frequency $\omega_1$ in the near-infrared region and a broadband white-light continuum pulse of frequency $\omega_2$ in the 450–750 nm region were used to irradiate the sample, and the TPA spectra in the $\omega_1 + \omega_2$ wavelength range were measured simultaneously using a multichannel detector. Two different $\omega_1$ wavelengths were chosen among the following wavelengths: 1300, 1400, 1450, 1750, and 1751 nm. The spectra measured with the different $\omega_1$ wavelengths were well overlapped when plotted against the $\omega_1 + \omega_2$ wavelength, which ensures that the observed spectra are due to the TPA process (Supplementary Figure S1). The polarizations of the $\omega_1$, $\omega_2$, and $\omega_1 + \omega_2$ lights were parallel to one another. The OPA spectra were measured using a commercial spectrometer (U-3310, Hitachi).

HBDI was synthesized according to the procedure reported in the literature [18]. The HBDI concentration was 9.53–37.0 μM for the OPA measurements and 5.1–30 mM for the TPA measurements. KOH aqueous solution was added to produce the anionic form. In acetonitrile and ethyl acetate solutions, 18-crown-6 was further added to assist the production of the anionic form. (Note that 18-crown-6 neither change the solvent characters nor interact with HBDI [19]). The concentrations of HBDI, KOH, and 18-crown-6 in each solution are summarized in the Supplementary Tables S1 and S2.

## 3. Results and discussion

The OPA spectra of HBDI in various solvents are shown with black lines in Figure 3. The $S_1 \leftarrow S_0$ absorption peak maxima of the neutral form vary only slightly: 370 nm (methanol), 369 nm (ethyl acetate), 367 nm (acetonitrile), 374 nm (DMF), and 376 nm (DMSO) (Figure 3a), whereas those of the anionic form change substantially, that is, 432 nm (methanol), 459 nm (ethyl acetate), 448 nm (acetonitrile), 465 nm (DMF), and 478 nm (DMSO) (Figure 3b). The different solvent dependence between the neutral and anionic forms has been reported previously by Tolbert and coworkers, who measured OPA spectra of HBDI in 16 different solvents [19]. They analyzed the solvatochromic behavior quantitatively, and concluded that the strong solvent dependence of the anionic form is due to its larger permanent dipole-moment difference between the $S_0$ and $S_1$ states compared with that of the neutral form.

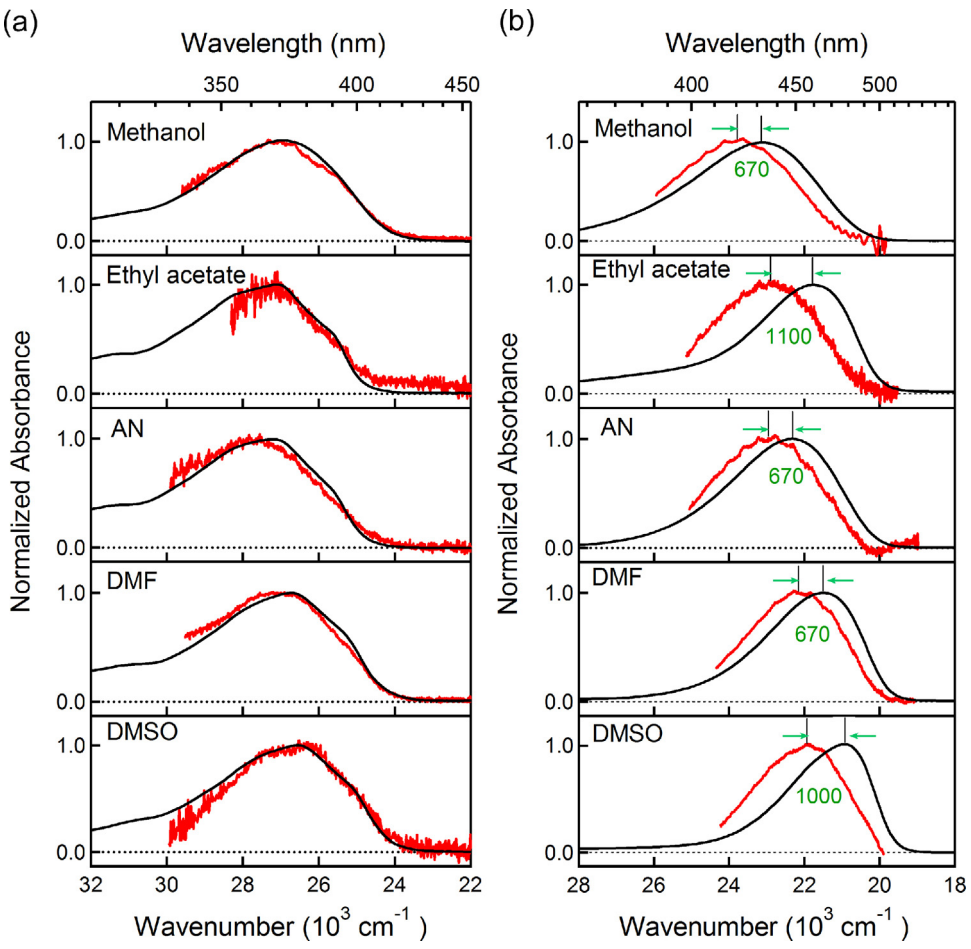


**Figure 3.** One-photon (black) and two-photon (red) absorption spectra of the (a) neutral and (b) anionic forms of HBDI in methanol, ethyl acetate, acetonitrile (AN), *N*,*N*-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO). The values in green denote the energy differences between the OPA and TPA peaks in units of wavenumbers.

The TPA spectra are displayed with red lines in Figure 3. As shown in Figure 3a, the OPA and TPA spectra of the neutral form are almost identical in each solvent within the experimental error. The coincidence between the OPA and TPA bands indicates that the neutral form is excited to the same $S_1$ state by either one-photon or two-photon excitation. In contrast, the TPA peaks of the anionic form of HBDI are significantly shifted to higher energy compared with the OPA peaks, not only in the case of HBDI in methanol reported before [15] but also in all solvents examined in this work: 420 nm (methanol), 437 nm (ethyl acetate), 435 nm (acetonitrile), 451 nm (DMF), and 456 nm (DMSO) (Figure 3b). Furthermore, the energy differences between the OPA and TPA peak maxima are largely dependent on the solvent: 670 $cm^{-1}$ (methanol), 1100 $cm^{-1}$ (ethyl acetate), 670 $cm^{-1}$ (acetonitrile), 670 $cm^{-1}$ (DMF), and 1000 $cm^{-1}$ (DMSO). The OPA and TPA absorption peak maxima of the neutral and anionic forms, and the energy differences between the OPA and TPA peak maxima of the anionic form are summarized in Table 1.

In the following, we consider the band shape (vibronic structure) of the OPA and TPA spectra using the vibronic theory. Particularly, we attempt to explain the relative blue shift of the TPA spectrum by considering only the vibronic transition to the $S_1$ state (Figure 2a), and show that the large solvent dependence of the magnitude of the blue shift cannot be rationalized.

In general, the $S_1 \leftarrow S_0$ OPA intensity is determined by the matrix element of the transition dipole moment $\mu(Q)$ at the nuclear coordinates $Q$ between the $S_0$ and $S_1$ states, $\langle S_1|\langle \nu_{S_1}|\mu(Q)|\nu_{S_0}\rangle|S_0\rangle$, where $|S_0\rangle$ and $|S_1\rangle$ are the electronic wavefunctions of the $S_0$ and $S_1$ states, respectively, and $|\nu_{S_0}\rangle$ and $|\nu_{S_1}\rangle$ are the vibrational wavefunctions of the $S_0$ and $S_1$ states, respectively. For the dipole-allowed $S_1 \leftarrow S_0$ transition, as in the case of HBDI, the OPA intensity is dominated by the term that involves the transition dipole moment at the equilibrium nuclear coordinate $Q_0$, $\mu(Q_0)$, and it is simply given by $\langle S_1|\mu(Q_0)|S_0\rangle\langle \nu_{S_1}|\nu_{S_0}\rangle$. This factor indicates that the vibronic structure of the OPA spectrum is determined by the Franck-Condon factor, $\langle \nu_{S_1}|\nu_{S_0}\rangle$ [20]. Similarly, the $S_1 \leftarrow S_0$ TPA intensity is determined by the matrix element of the TPA tensor $S(Q)$ between the $S_0$ and $S_1$ states, $\langle S_1|\langle \nu_{S_1}|S(Q)|\nu_{S_0}\rangle|S_0\rangle$. If the electronic part of the TPA tensor does not depend on $Q$, the TPA intensity is simply given by the term, $\langle S_1|S(Q_0)|S_0\rangle\langle \nu_{S_1}|\nu_{S_0}\rangle$. Thus, the vibronic structure of the TPA spectrum is also determined by the Franck-Condon factor $\langle \nu_{S_1}|\nu_{S_0}\rangle$ [21,22]. As a result, the vibronic structure of the $S_1 \leftarrow S_0$

**Table 1**
The OPA and TPA absorption peak maxima of the neutral and anionic forms, $\lambda_{max}$, and the energy differences between the OPA and TPA peak maxima of the anionic form.

| Solvent | Neutral | | Anion | | |
|---|---|---|---|---|---|
| | $\lambda_{max}$ (nm) | | $\lambda_{max}$ (nm) | | Energy difference ($cm^{-1}$) |
| | OPA | TPA | OPA | TPA | |
| Methanol | 370 | 369 | 432 | 420 | 670 |
| Ethyl acetate | 369 | 365 | 459 | 437 | 1100 |
| Acetonitrile | 367 | 360 | 448 | 435 | 670 |
| DMF | 374 | 368 | 465 | 451 | 670 |
| DMSO | 376 | 374 | 478 | 456 | 1000 |

absorption in both the OPA and TPA spectra are identical, which does not give any blue shift of the TPA spectrum. This argument does not accord with the observed differences in the OPA and TPA spectra.

The blue shift of the TPA spectrum can be theoretically explained by considering the $Q$-dependence of the TPA tensor. Particularly, for non-centrosymmetric molecules like HBDI, the TPA tensor can be approximated as $\boldsymbol{S}(Q) \approx \boldsymbol{\mu}(Q) \times \Delta\mu$, where $\Delta\mu$ is the difference in the permanent dipole moments of the $S_0$ and $S_1$ states [23]. Recently, Drobizhev et al. reported a physical model in which the series expansion of $\Delta\mu(Q)$, to the linear term with respect to $Q$, gives rise to the $Q$-independent (Franck-Condon) and $Q$-dependent (Herzberg-Teller) terms and claimed that the both terms contribute together to the band shape of the TPA spectrum [17]. The $Q$-independent term gives the vibronic structure determined by the Franck-Condon factor, $\langle v_{S_1}|v_{S_0}\rangle$, while the $Q$-dependent term gives a blue-shifted component originating from the $\langle v_{S_1}|Q|v_{S_0}\rangle$ factor. In other words, the band shape of the TPA spectrum can be represented by a sum of two contributions: one component that has the same vibronic structure as the OPA spectrum and the other component that is blue-shifted from the OPA spectrum by the vibrational energy of the relevant mode. Thus, in their model, the energy difference between the OPA and TPA peak maxima reflects the vibrational energy(ies) of the relevant mode(s) ($Q$). It should be noted that, since the transition dipole moment $\mu(Q)$ is commonly involved in both the OPA and TPA processes, its $Q$-dependence is not expected to give a significant blue shift.

Based on their model, the energy difference between the OPA and TPA peak maxima should be almost the same in all the solvents, because the solvent dependence of the vibrational energy is very small in general. For example, the frequency of the most intense Raman band of the anionic form of HBDI (the C=N stretching mode of the imidazolinone ring) is observed at 1550 $cm^{-1}$, 1555 $cm^{-1}$, 1556 $cm^{-1}$, 1556 $cm^{-1}$, and 1556 $cm^{-1}$ in DMSO, 2-propanol, methanol, ethanol, and water, respectively [24–26]. However, as clearly observed in the present study, the peak energy differences between the OPA and TPA spectra drastically change from 670 $cm^{-1}$ to 1100 $cm^{-1}$ by solvent. This observation is not consistent with the argument that the blue shift originates from the $\langle v_{S_1}|Q|v_{S_0}\rangle$ factor. We also confirmed that the experimental blue shift cannot be accounted for by changing the relative amplitudes of the $Q$-dependent and $Q$-independent terms while keeping the vibrational energy constant: First, we calculated a sum of the experimental OPA spectrum and another spectrum that is obtained by displacing the experimental OPA spectrum toward the high frequency side, and then fitted the sum to the TPA spectrum measured in methanol. The vibrational energy determined in this fitting is 800 $cm^{-1}$ for the methanol solution. Next, we attempted to reproduce the TPA spectrum measured in ethyl acetate by the same procedure with the common 800 $cm^{-1}$ displacement. However, we failed to reproduce it even though we thoroughly changed the relative amplitude of the two terms (Supplementary Figure S2). Therefore, the strong solvent dependence of the magnitude of the blue shift is inconsistent with the argument that the TPA blue shift results from the $\langle v_{S_1}|Q|v_{S_0}\rangle$ factor, and hence opposes the assignment that TPA arises from the transition to the vibrationally excited $S_1$ state. This conclusion holds regardless that the relevant vibrational mode is one or more.

Alternatively, the involvement of the closely lying $S_2$ state readily rationalizes the experimental observation, where the $S_2$ state has smaller one-photon absorptivity and larger two-photon absorptivity than the $S_1$ state. Then, in the OPA spectrum, the $S_2 \leftarrow S_0$ transition band is hidden by the stronger $S_1 \leftarrow S_0$ transition band, and the OPA peak maximum is close to that of the $S_1 \leftarrow S_0$ transition band. Inversely, in the TPA spectrum, the intensity of the $S_2 \leftarrow S_0$ transition band is stronger than that of the $S_1 \leftarrow S_0$ band, and the TPA peak maximum predominantly reflects the $S_2 \leftarrow S_0$ transition. This makes the TPA band appear shifted to the shorter-wavelength side compared with the OPA band. As is well-known, the electronic states having different characters gain substantially different stabilization energy in different solvents. We consider that solvent dependence of the peak energy difference observed in the present study arises from difference in solvatochromic shift of the $S_1 \leftarrow S_0$ and $S_2 \leftarrow S_0$ transitions.

It should be mentioned that Olsen et al. performed a SA3-CAS calculation and reported the existence of a dark excited state of the anionic form of 4′-hydroxybenzylidene imidazolinone (HBI), a simpler model compound of the GFP chromophore [27]. Specifically, they predicted two excited states: a lower-energy bright ($S_1$) state and a higher-energy dark ($S_2$) state. Although the calculated $S_2 \leftarrow S_0$ transition energy was notably higher than that observed in the present study, the dominant electronic character of the $S_2$ state was found to be a doubly excited configuration. The doubly excited configuration is characterized by one-photon forbidden and two-photon allowed nature, which matches the character of the $S_2$ state suggested by the present study.

Our conclusion for HBDI is also applicable to eGFP. We thus propose the mechanism for two-photon fluorescence of eGFP as follows. When the anionic form of eGFP is two-photon excited, the $S_2$ state is initially populated, after which immediate relaxation to the $S_1$ state occurs. The $S_1$ state then exhibits fluorescence identical to that observed by one-photon excitation. We note that different one- and two-photon fluorescence excitation spectra have also been reported for the anionic forms of orange and red FPs [13]. However, the results of these previous studies do not necessarily suggest that the corresponding $S_2$ state exists for these FPs. The chromophores of these FPs are different from HBDI, and hence the mechanism would be different for each FP. Further TPA measurements of various FPs and corresponding model chromophores are desirable to fully understand the relevant electronic structure of the excited states as well as the fluorescence mechanism after two-photon excitation.

## 4. Conclusion

We measured the TPA spectra of HBDI in various organic solvents. The TPA bands of the anionic form of HBDI were shifted to higher energies compared with the OPA bands in all of the investigated solvents. Moreover, the energy difference between the OPA and TPA bands changed substantially from 670 $cm^{-1}$ in methanol to 1100 $cm^{-1}$ in ethyl acetate. We discussed the origin of the blue shift of the TPA band on the basis of the strong solvent dependence and concluded that it is not due to a transition to the vibrationally excited $S_1$ state. The difference between the OPA and TPA spectra very likely arises from the participation of the $S_2$ state. It was considered that the stronger $S_2 \leftarrow S_0$ and the weaker $S_1 \leftarrow S_0$ transition bands are indistinguishably overlapped in the TPA spectrum. This conclusion is also applicable to eGFP and suggests the involvement of the $S_2$ state in the two-photon fluorescence of eGFP.

## Acknowledgements

We are grateful to Dr. Atsushi Miyawaki at the Brain Science Institute, RIKEN, Japan and Dr. Hideaki Mizuno at Katholieke Universiteit Leuven, Belgium for discussions. This work has been supported by a Grant-in-Aid for Scientific Research (A) (No. 25248009) and a Grant-in-Aid for Scientific Research (C) (No. 24550033) from the Japan Society for the Promotion of Science (JSPS), and a Grant-in-Aid for Scientific Research on Innovative Areas (No. 25104005) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cplett.2015.04.028

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