論文 / 著書情報
Article / Book Information

題目(和文)	電子ドナーおよびアクセプター基を有するピレン誘導体の合成と光物理的性質
Title(English)	Synthesis and Photophysical Properties of Functional Pyrene Derivatives with Electron Donor and/or Acceptor Groups
著者(和文)	仁子陽輔
Author(English)	Yosuke Niko
出典(和文)	学位:博士（工学）, 学位授与機関:東京工業大学, 報告番号:甲第9759号, 授与年月日:2015年3月26日, 学位の種別:課程博士, 審査員:小西　玄一,和田　雄二,安藤　慎治,ﾊﾞｯﾊ　ﾏｰﾃｨﾝ,戸木田　雅利
Citation(English)	Degree:, Conferring organization: Tokyo Institute of Technology, Report number:甲第9759号, Conferred date:2015/3/26, Degree Type:Course doctor, Examiner:,,,,
学位種別(和文)	博士論文
Category(English)	Doctoral Thesis
種別(和文)	要約
Type(English)	Outline

博士論文要約

Synthesis and Photophysical Properties of Functional Pyrene Derivatives with Electron Donor and/or Acceptor Groups

（電子ドナーおよびアクセプター基を有するピレン誘導体の合成と光物理的性質）

Yosuke Niko

2015

Department of Organic and Polymeric Materials

Tokyo Institute of Technology

General Introduction

1. Organic fluorescent molecules

In recent decades, organic fluorescent molecules have attracted considerable interest because of their potential as light-emitting materials in organic electronics, biology, and medical science. They have been used extensively as fluorescent probes and sensors because they can exhibit ***high sensitivity***, ***selectivity***, and ***rapid response***, which, in combination with fluorescence spectroscopy and microscopy, enables the detection and visualization of pico- and even femtomolar amounts of target materials in samples of interest, with real-time monitoring in picosecond timescales. These characteristics make these molecules extremely useful for monitoring the local structure, dynamics, function, and chemical and/or biological events in samples, whether the subjects of study are small or macro molecules, living or non-living subjects, or homogeneous or heterogeneous systems. Due to these qualities, fluorescence techniques are becoming irreplaceable research tools.

As highlighted by the awarding of recent Nobel Prizes to Shimomura, Chalfie, and Tsien (2008) for the discovery of green fluorescent protein (GFP), and to Hell, Betzig, and Moener (2014) for the development of super-resolved fluorescence microscopy, the development and application of optical techniques related to fluorescence has always been accompanied by the discovery of new fluorophores and/or phenomena. Furthermore, increasing challenges in each research area, and the desire for improvement of optical components, have also stimulated research in this field (Figure 1). Unsurprisingly, in this cycle, the performance requirements for fluorophores, *e.g.*, brightness, color, emitting principle, environment responsiveness, and stability against heat and photo-irradiation, have changed and often become much greater.

In summary, it remains necessary to develop new fluorescent molecules and to explore new fluorescence phenomena through study of the structure/property relationships in new molecules.

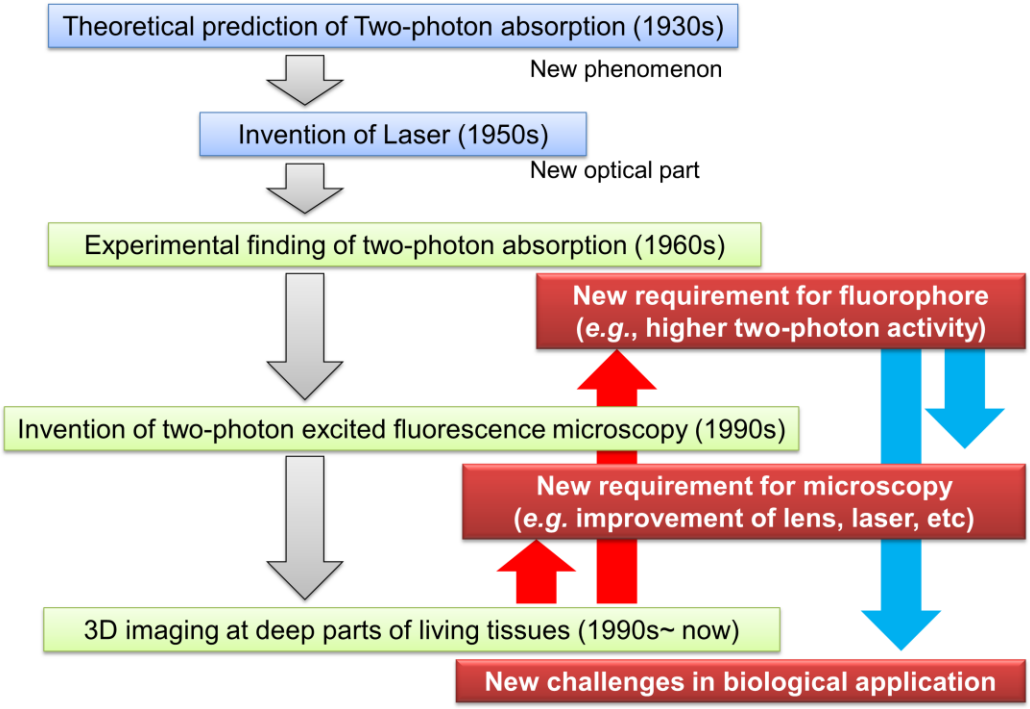

Figure 1. Flow chart for the history of two-photon excited fluorescence techniques as an example of the relationship between fluorophore development, optical techniques, and applications in their progress.

2. Biomedical applications of organic fluorescent molecules

Among the many applications of organic fluorophores, their use in biomedical fields as fluorescent probes has played an important role. This is because, in addition to the three above-mentioned characteristics, fluorescent probes can work as diagnostic and/or contrast agents, and can realize "***non-invasive" in situ***, ***in vivo, and/or in vitro imaging*** in combination with fluorescence techniques (see section 3). Furthermore, they display good biocompatibility, as opposed to inorganic emitters such as quantum dots and dye-doped silica nanoparticles. In fact, they have been applied to a wide range of biomedical functions such as the detection of specific biochemical species, the measurement of ion concentrations inside living cells, the study of protein folding dynamics, the measurement of distances within macromolecules and biological assemblies, the study of membrane structure and function, the monitoring of ligand binding to biochemical species, the study of drug interactions with cell receptors, the detection of DNA single-base mismatches, and the analysis of hole transfer kinetics in DNA. The variety in the specifications of fluorophores has increased in response to the diversity of their applications, demonstrating their versatility. A more detailed example related to this thesis is described below.

Fluorescent probes for lipid rafts in plasma membranes

In 1997, Simon and Ikonens[1] proposed the lipid microdomain hypothesis, which proposed that the plasma membrane is a complex system comprising a combination of a liquid ordered (Lo) phase and a liquid disordered (Ld) phase consisting of saturated and unsaturated lipids, respectively, along with cholesterol. In other words, rigid Lo compartments enriched with sphingolipids and cholesterol float in the Ld phase enriched with phospholipids. Because of this, such rigid compartments are usually called lipid "rafts," and are implicated in many cellular processes such as signal transduction, pathogen invasion, cholesterol homeostasis, neurodegenerative disease, and angiogenesis,[2] which were difficult to explain by the classical "fluid mosaic" model. Therefore, much effort has focused on understanding the roles of lipid rafts. Consequently, the visualization of this domain remains an important challenge. According to a recent review by Klymchenko (2014),[3] the use of fluorescent probes is the only way to visualize lipid rafts, not only in model membranes, but also in living cells. In fact, his group succeeded in visualizing both the Lo and Ld phases of model membrane in different colors by using a Nile Red derivative, an environmentally sensitive fluorophore (Figure 2),[4] and subsequently demonstrated the great possibility of the presence of cholesterol-rich Lo phase in living cell membrane.

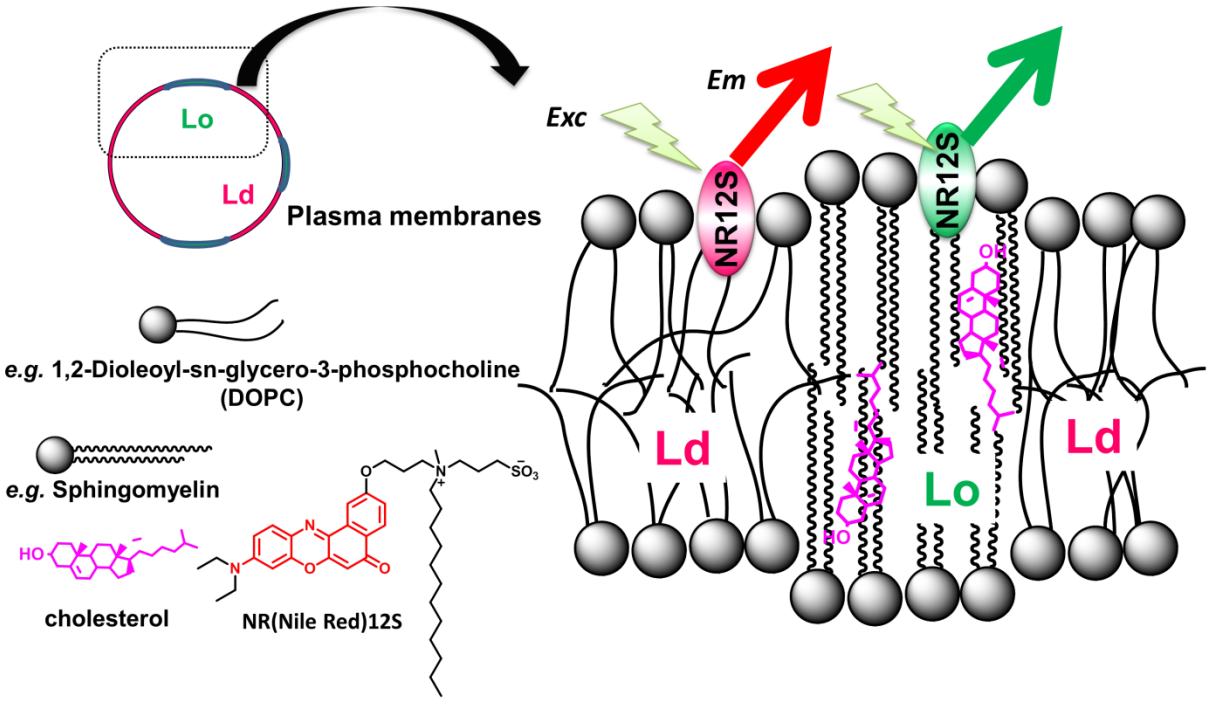

Figure 2. Visualization of lipid rafts with the Nile Red derivative, NR12S.

However, to perform more detailed investigations through visualization, further improvements to the fluorescent probes are required. For instance, while Nile Red can be used for quantitative analysis by solvatochromism, its photostability against excitation light is insufficient for prolonged imaging. Alternatively, if and super-resolved imaging is required (see section 3.3), the probes must possess ultra-brightness and photostability. This situation clearly illustrates that the selection and development of fluorescent probes should be performed taking research-purpose and available instrumentation into account.

3. Fluorescence techniques

The combination of fluorescent probes with fluorescence spectroscopy and/or microscopy has led to the powerful and non-invasive techniques enabling high sensitivity, high selectivity, and rapid response monitoring of the target samples, as mentioned above. In addition, such fluorescence techniques do not require special handling and rely on relatively inexpensive hardware compared to other methods such as magnetic resonance imaging (MRI), X-ray, positron emission tomography (PET), or single-photon emission computed tomography (SPECT).[5] Over the past 20 years, fluorescence techniques, especially microscopy techniques, have progressed dramatically. Some examples are described below.

3.1. Confocal laser scanning microscopy (CLSM)

When people talk about "fluorescence microscopy," they most commonly refer to CLSM. The development of an efficient and affordable laser light source popularized this type of microscopy. In this system the positions of the light source, sample, and image are conjugated, which is called as confocal optical system.[6] Therefore, CLSM provides better-resolved images of plane surfaces than normal fluorescence microscopy. In addition, CLSM can provide images of depth direction of samples, though its resolution and depth are much lower than those of two-photon excited fluorescence microscopy (TPFM, see next section). Furthermore, CLMS has the advantage of employing inexpensive hardware. Conversely, the disadvantage of CLMS is that, in principle, even non-recorded samples must be exposed to continuous laser excitation, which may damage both the fluorophore and samples. Therefore, the fluorophore for CLMS should ideally have high photostability. Furthermore, longer excitation wavelengths located around the visible or near infrared region, and compatibility with limited laser sources, *e.g.*, 405 nm, 445 nm, 488 nm, 515 nm, and 640 nm diode lasers, are also desirable.

3.2. Two-photon excited fluorescence microscopy (TPFM)[7]

This microscopy technique utilizes simultaneous "two-photon absorption" by fluorophores, which is one of the third-order, non-linear optical phenomena. TPFM has an advantage of exciting only the fluorophores at the focal point of laser excitation. Therefore, its spatial resolution is very good. Moreover, fluorophores can usually be excited by long wavelengths located in the biological window (650-1100 nm),[8] which allows the imaging of areas of cells and tissues micro- and even millimeters deep. Furthermore, because the fluorophores outside of the focal point are not excited, the samples and fluorophores remain mostly undamaged. The disadvantages of TPFM are lower plane resolution than one-photon

microscopy due to the use of light with twice the wavelength, the difficulty in handling the Ti-sapphire laser, and expensive hardware. Furthermore, the number of fluorophores that exhibit sufficient two-photon absorption is limited. Although the next-generation fiber laser is expected to solve the problems of cost and handling, the number of fluorophores compatible with this laser source are rarer still.[9]

CLMS and TPFM can be used with **fluorescence correlation spectroscopy (FCS)**,[10] which can provide not only static information (fluorescence intensity) but also dynamic information (the number of molecules, shape, and diffusion kinetics) from the samples of interest.

3.3. Super-resolved microscopy[11]

Recently, several fluorescence microscopy techniques that can overcome the diffraction limit of light and realize super-resolved imaging, even to tens of nanometers, have been developed, and have paved the way for single-molecule fluorescence imaging. For example, stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM), and stochastic optical reconstruction microscopy (STORM) are considered next-generation fluorescence techniques. As one might expect, single-molecule fluorescence imaging requires ultra-bright fluorophores. In addition, when STED is used, the fluorophores should be sufficiently photostable to withstand intense STED light. Moreover, PALM/STORM requires special fluorophores that can switch between fluorescence “on” and “off” states.

Additionally, there exist microscopy techniques based on the principle of fluorescence lifetime or second-harmonic generation, *i.e.*, fluorescence lifetime imaging (FLIM)[12] and second-harmonic imaging microscopy (SHIM).[13]

Each of the microscopy techniques discussed above possess inherent advantages and disadvantages. Therefore, the nature of a given problem should determine which technique is used. Furthermore, the performance of each microscopy technique strongly depends on the probe molecules.

4. Classification of synthetic organic fluorescent molecules

Much research effort has focused on the development of new organic fluorescent molecules to address the requirements of the applications and instrumentation used. In this section, several synthetic organic fluorophores are classified according to their characteristics, namely 1) highly bright and photostable fluorophores, 2) environment-responsive fluorophores, and 3) two-photon active fluorophores. Natural fluorophores, like tryptophan, and fluorophores with specific properties/functions such as water solubility, active targeting of proteins, and supramolecular assembly formation, which can all be introduced through synthetic organic methods, are beyond the scope of this work.

4.1. Highly bright and photostable fluorophores

Fluorophores discussed in this section are brighter and more photostable than the environment-responsive dyes. Therefore, they are suitable for not only dyeing, but also for single-molecular imaging. However, they are usually unsuitable for quantitative analysis, although a few exceptions exist.

4.1.1. Classical synthetic fluorophores

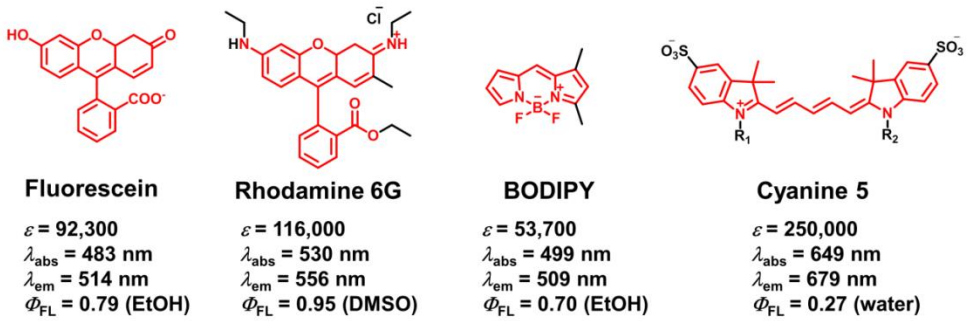

Figure 3. Chemical structures of "classical" synthetic fluorophores. The data for Fluorescein, Rhodamine 6G, BODIPY, and Cyanine 5 were taken from references [14], [15], [16], and [17], respectively.

Fluorescein: Fluorescein is one of the most well-known and widely used fluorophores, and was developed by Bayer in 1871. As evinced by the extinction coefficient and fluorescence quantum yield data given in Figure 3, fluorescein is highly bright. In addition, its absorption and fluorescence are pH-dependent in the range ~5–7.[14c] A variety of fluorescein derivatives such as fluorescein isothiocyanate have been developed.

Rhodamine: Rhodamine is an amino-containing analog of fluorescein and, like fluorescein, it is well-known and widely used. Several derivatives have been developed, including

Rhodamine 6G, Rhodamine B, and Rhodamine 123. Many members of this family possess high extinction coefficients and fluoresce from the yellow to the red region with high fluorescence quantum yields. Furthermore, rhodamine derivatives display lower dependence on pH and higher photostability than fluorescein.

BODIPY: BODIPY was first developed by Treibs and Kreuzer in 1968,[16a] and, as with fluorescein and rhodamine, a variety of analogs have been developed. The characteristics of these dyes are a small Stokes shift, a narrow fluorescence band width, and lower environmental sensitivity to factors such as polarity. The majority of the members of this family are highly bright, and new derivatives are still being developed.

Cyanine: Cyanine and its derivatives have a structure based on a polymethine chain with two terminal amino/imino groups. The most widely used cyanine fluorophores contain heterocyclic rings that increase stability and simplify synthesis. This family is characterized by the polymethine chain length. Notably, cyanine 5 possesses an extremely high extinction coefficient and exhibits near-infrared emission. Therefore, it is considered a super-bright fluorophore. Its photostability is also high, so it is widely used for single-molecular fluorescence imaging.

The derivatives of all the dyes discussed above have been developed in order to achieve specific characteristics such as desired absorption/fluorescence wavelengths and water solubility for particular applications. This has led to a wide variety of derivatives being commercially available.

4.1.2. π-Extended aromatic hydrocarbons

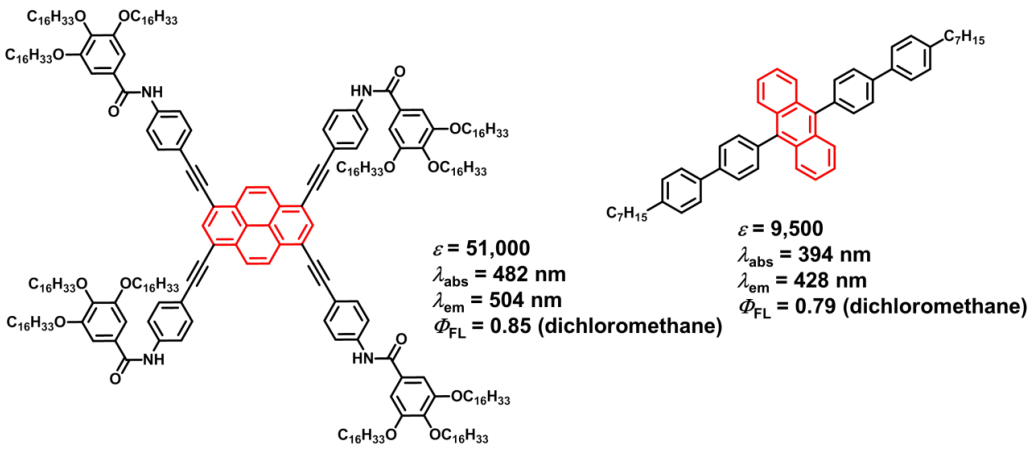

Figure 4. Examples of π-extended aromatic hydrocarbons. The data for pyrene (left) and anthracene (right) derivatives were taken from references [18] and [19], respectively.

Aromatic hydrocarbon chromophores such as naphthalene, fluorene, anthracene, pyrene, and perylene are very often derivatized by π-extension with phenyl, vinyl, and ethynyl groups. These derivatives show high extinction coefficients (occasionally exceeding 100,000) and high quantum yields (often exceeding 0.90) in comparison with the parent fluorophores. They usually possess rigid structures, which imbue thermal stability, and exhibit blue emission. Therefore, the majority of their applications are not in bio-imaging, but as emitting layers in organic light-emitting diodes (OLEDs). Therefore, investigations into the solid-phase emission of these fluorophores has been widely conducted.[20]

4.2. Environment-sensitive dyes

Nearly all organic fluorophores show a greater or lesser dependence on some sort of environmental condition. For example, typical fluorophores show slightly red-shifted fluorescence with increased solvent polarity owing to the larger dipole moment in the excited state, and proceeding solvent relaxation. Similarly, they usually become less fluorescent at higher temperatures owing to accelerated thermal vibronic deactivation. Such effects may be considered general environmental effects because they are also observed in the molecules in section 4.1. This section introduces fluorophores whose photophysical properties are affected much more by environmental conditions than by general effects, and subsequently categorizes them by the differences in their fluorescence mechanisms.

4.2.1. Polarity-sensitive dyes (1)

Ham effect: Pyrene is the most well-known chromophore that exploits the Ham effect. The fluorescence spectrum of pyrene is highly structured and the intensity of each vibronic structure is sensitive to solvent polarity. Therefore, the intensity ratio between two specific vibronic structures enables us to determine the local polarity surrounding pyrene (Figure 5a). This property is often used to determine the critical micelle concentration of unknown detergents.[21a]

Aromatic carbonyl compounds: Many π-systems possessing formyl, ketone, imino, and *β*-diketone groups are known to form n–π* first singlet states ($S_1(n–\pi^*)$) after photoexcitation, owing to the *n* electron on the oxygen or nitrogen of the carbonyl or imino group, respectively. As a result, such dyes are less fluorescent, owing to enhancement of the intersystem crossing constant (see El-Sayed's rule). Conversely, in protic solvents they become fluorescent because hydrogen bonds between solvent molecules and the carbonyl groups, *i.e.*, the H-bond acceptor groups, increase the energy level of the n–π* excited state ($S_n(n–\pi^*)$) and generate the fluorescent $S_1(\pi–\pi^*)$ state. This property enables examination of the hydration level of the

dyes.[22a] In addition, when an imino group or β-diketone is used, it is also possible to detect a metal ion by the same principle.[23]

The energy level of $S_2(n–\pi*)$ in 2-formylnapthalene, 9-formylanthracene, and 1-formylpyrene lies close to that of $S_1(\pi–\pi*)$. In this case, the mixing between both/either $S_1(\pi–\pi*)$ and $S_2(n–\pi*)$ or $S_1(\pi–\pi*)$ and $T_2(n–\pi*)$ will occur due to out-of-plane vibration. The former causes the dye to undergo internal conversion, the so-called proximity effect,[24] and the latter causes the dye to undergo intersystem crossing, as illustrated in Figure 5b.

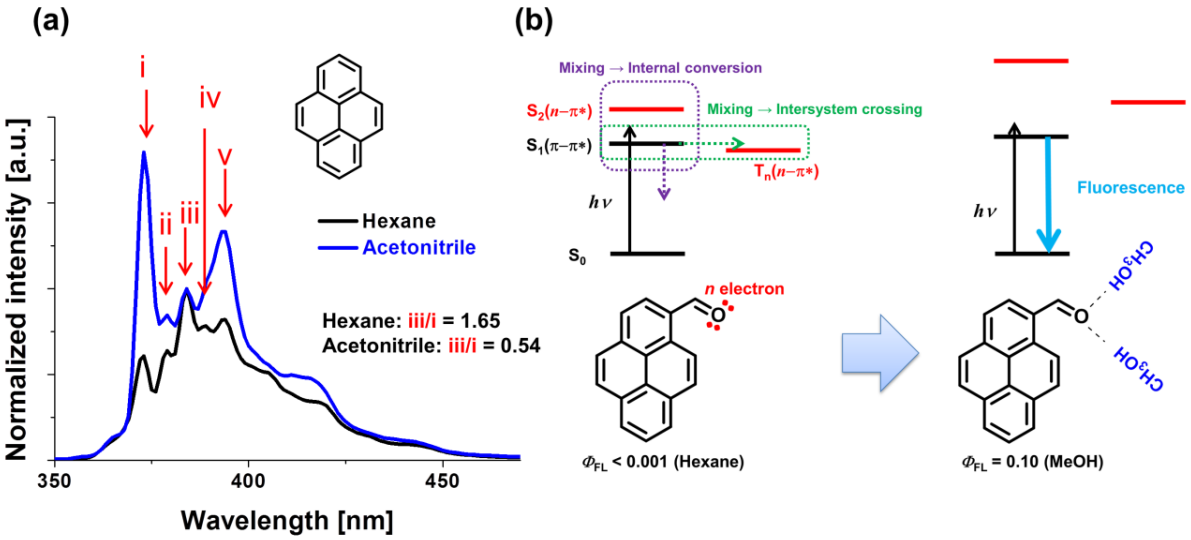

Figure 5. (a) Ham effect of pyrene and (b) fluorescence mechanism of 1-formylpyrene. The data for (a) and (b) were taken from references [21b-c] and [22b], respectively.

4.2.2. Polarity-sensitive dyes (2) "Fluorescence solvatochromism"

Excited-state intramolecular charge transfer (ESICT) system: A typical solvatochromic fluorophore based on the principle of ESICT is Prodan, developed by Weber and Farris in 1979.[25] It exhibits strongly red-shifted fluorescence when solvent polarity is increased. This is because electronic excitation dramatically enhances the dipole moment in excited state due to intramolecular charge transfer (ICT) from the dialkyl amino group to the carbonyl group (Figure 6). In response to changes in solvent polarity, the fluorescence wavelength, intensity, and lifetime also change. These characteristics are very useful for investigating the local polarity around probes, and for performing ratiometric studies, as discussed in section 2. The fluorescence properties of ESICT-based dyes are affected not only by dipole-dipole interactions between solvent and solute, but also by H-bonding. This is because nearly all dyes in this class possess a carbonyl group, which is an H-bond acceptor. Therefore, in protic

solvents or H-bond donor solvents such as chloroform, the spectral shifts are often larger than those predicted from the dielectric constants alone of the solvent, because of the additional effect of H-bonding. This is also important for analyzing the quantity of water (a strong H-bonding donor medium) around the probes.

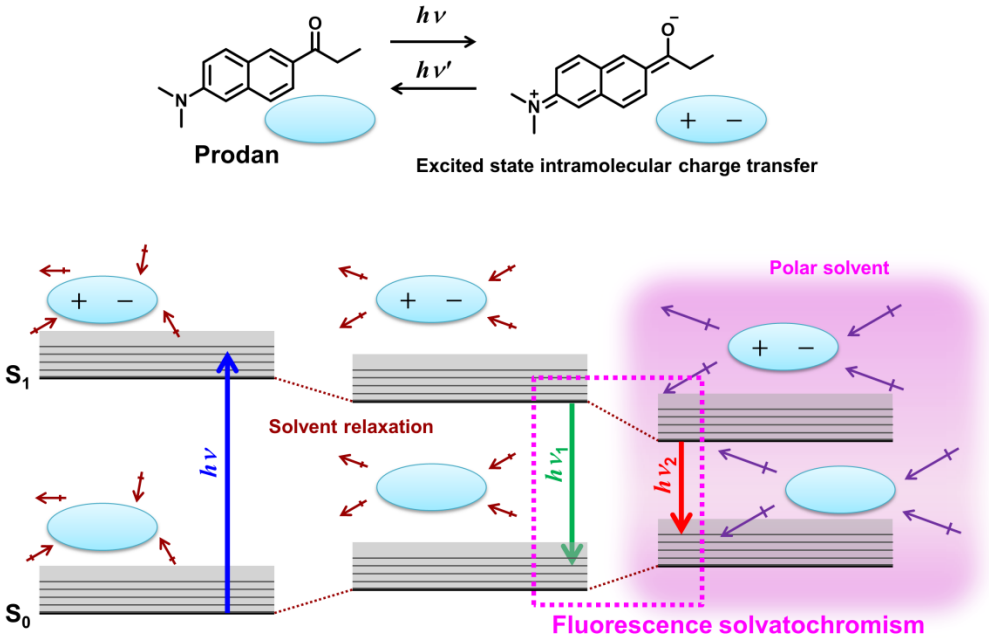

Figure 6. Energy diagram for fluorescence solvatochromism where the fluorophore has a small dipole moment in the ground state and a large dipole moment in the excited state, as seen in ESICT-type fluorophores like Prodan. A detailed discussion of this phenomenon is presented in section 5.

Among the fluorophores in this class, Prodan, FR0, and Nile Red should be highlighted (Figure 7). Prodan possesses well-balanced fluorescence properties in terms of solvatochromic response, fluorescence quantum yield, and molecular size, and is therefore still used in several applications. In addition, Prodan is a so-called "D-π-A," or "push-pull," dye, and therefore has been an important model for subsequent D-π-A dyes. In fact, FR0 was developed as an analog of Prodan (Klymchenko, 2010),[26] and is arguably the best of the Prodan analogs as it represents a significant improvement over Prodan in terms of brightness, photostability, two-photon activity, and absorption and fluorescence wavelengths. Nile Red[4,27] possesses higher photostability and further red-shifted absorption and fluorescence compared with other existing D-π-A dyes.

Except for a few examples, like Nile Red, most Prodan-type ESICT-based

solvatochromic fluorophores have the disadvantage of displaying absorption wavelengths in the UV or violet region. This may damage biological tissues, and the transparency of the excitation light in this region is insufficient. Furthermore, appropriate excitation sources are also limited. This is not the case for Nile Red. However, Nile Red's solvatochromic response is weaker than those of Prodan and FR0. Moreover, compared to the dyes in section 4.1., the extinction coefficients and photostabilities of Prodan analogs are very low. Therefore, imaging using these dyes may require the use of an excess amount of the fluorophore. In addition, unexpected reactive species, which may damage the samples of interest, may be generated through the dye's degradation process, as further discussed in section 5.

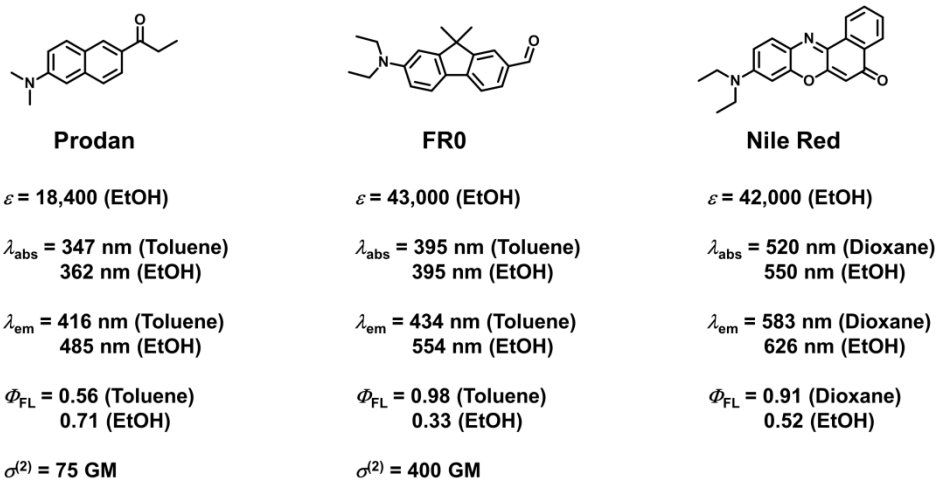

Figure 7. Chemical structures of Prodan, FR0, and Nile Red. The data for Prodan and FR0 were taken from reference [26]. The data for Nile Red were taken from reference [4].

Excited-state intramolecular proton transfer (ESIPT) system: ESIPT is a phenomenon in which a proton transfers between groups, usually from a hydroxyl proton donor to a carbonyl proton acceptor, in the same excited state molecule (Figure 8). The main governing force for ESIPT is the change in the acidity and basicity of the proton donor and acceptor, respectively. When ESIPT occurs, a more stable excited tautomer, (T*), is generated, and therefore, fluorescence from this species is observed at longer wavelengths than from a normal excited species (N*). Historically, ESIPT was observed in the fluorescence of methylsalicylate by Weller in 1950s.[28] This fluorophore and its derivatives show only single fluorescence band from either N* or T* because of the low energy barrier and large energy difference between them. Since then, 3-hydroxyflavone (3HF) has been developed (Frolove, 1974),[29] and is probably the most important chromophore in this class as it can exhibit dual fluorescence

bands from both N* and T*. The intensity ratio of the fluorescence from these two species (I_{N*}/I_{T*}) depends on the solvent polarity and H-bond donor ability. Most importantly, this characteristic simultaneously provides four parameters, ν_{abs}, ν_{N*}, ν_{T*}, and I_{N*}/I_{T*}, which help realize better quantitative analyses than do ESICT dyes. (ν_{abs}, ν_{N*}, and ν_{T*} denote wavenumber of absorption, fluorescence form normal state and tautomer, respectively.)

The drawbacks of this class of fluorophore are similar to those of ESICT dyes, *i.e.*, short absorption wavelengths, low extinction coefficients, low photostability, and potentially lower flexibility in molecular design than in ESICT dyes. In addition, the fluorescence quantum yields of many ESIPT dyes are lower than those of ESICT dyes.

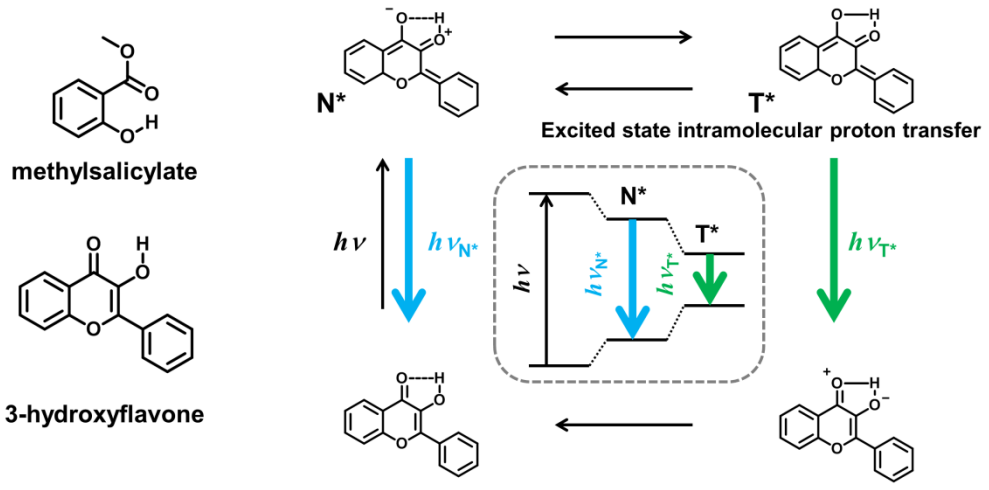

Figure 8. Chemical structures of methylsalicylate and 3-hydroxyflavon, and the energy level diagram for ESIPT.

4.2.3. Viscosity-sensitive dyes "Molecular rotors"

The fluorophores known as "molecular rotors" alter their fluorescence intensities in response to environmental viscosity. For example, DCVJ (Loutfy, 1982, Figure 9)[30] usually displays low fluorescence. This is because intramolecular rotation derived from the dicyanovinyl moiety induces internal conversion and subsequent deactivation, known as the free-rotor effect. However, in viscous media, intramolecular rotation is suppressed, and DCVJ becomes more fluorescent. For this reason, molecular rotors are useful for measuring the microviscosity surrounding the dyes. Moreover, if a molecular rotor possesses a D-π-A structure, it can exhibit sensitivity to solvent polarity, as seen in CL2 (Cho, 2008)[31] where the chalcone structure plays similar role to the dicyanovinyl group in DCVJ.

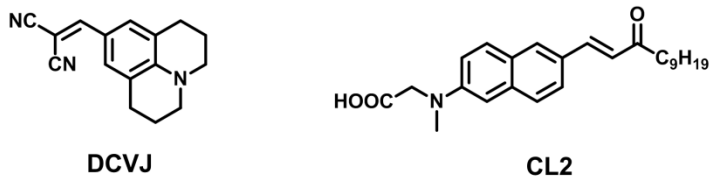

Figure 9. Chemical structures of two molecular rotors.

4.2.4. Concentration effect

Excimer: Pyrene is the most well-known chromophore which forms excimers, *i.e.*, excited state dimers, because it has a sufficiently lengthy singlet lifetime for excimer formation.[32] If the concentration is sufficiently high, excited pyrene easily forms a complex with a neighboring pyrene molecule in the ground state (Figure 10a). The formed excimer is more energetically stable than a monomer, and, therefore, shows longer fluorescence wavelengths. Although an excimer is typically formed by dynamic processes, as described above, there also exist static mechanisms, as demonstrated by pyrenophane. By using the change in fluorescence between pyrene and its excimer, quantitative study of the dynamics of macromolecular and/or biological assemblies can be performed.[33]

Concentration-caused quenching: Typically, fluorescent molecules become much less fluorescent in high concentrations owing to H-aggregation. This quenching is a prominent problem for OLEDs and ultra-bright organic nanoparticles. To overcome this problem, several approaches, including introduction of bulky substituents and/or counterions into the fluorophore, have been developed. It is worth noting, though, that this quenching principle is often used in the detection of supramolecular assemblies, based on similar principles to the Ham effect and excimer formation.

The following dyes are also environment-sensitive. However, such characteristics are generally used for the development of ultra-bright emitters, rather than qualitative analysis, like with ESICT and ESIPT dyes.

Aggregation-induced enhanced emission (AIEE): In contrast to typical fluorophores, there exist fluorophores that become highly fluorescent in high concentrations and aggregated states. Tang *et al.* have developed this type of molecule and described a clear molecular design concept for them. In most cases, such dyes possess twisted π-systems as illustrated in Figure 10b.[34] Furthermore, they developed AIEE fluorophore-based ultra-bright nanoparticles and applied them to *in vivo* imaging.

H- and J-aggregation: When the distance between fluorophores is small enough to allow interaction, the absorption spectrum of the fluorophore itself shifts towards either the blue or the red region. This phenomenon can be explained through the molecular exciton model proposed by McRae and Kasha in 1964.[35] In this model, only the interaction of transition dipoles between the nearest molecules is considered, and this model hypothesizes that all transition dipoles in one aggregate state are parallel. If the angle between the direction of the dipoles and the line drawn through the center of all dipoles is 0°, this aggregate is called a perfect J-aggregate. If the angle is 90°, the aggregate is called a perfect H-aggregate. The original energy state of the fluorophore splits into two new states owing to the interaction of such dipoles in the aggregate state. In a J-aggregate, the energy level of a newly generated allowed transition state is lower than that of a forbidden transition state. Therefore, J-aggregated fluorophores show red-shifted absorptions and enhanced fluorescence. These phenomena observed in J-aggregation are in complete contrast to those in H-aggregation. To the best of my knowledge, J-aggregation occurs usually in the thin film and nanofiber, not organic nanoparticles in solution. Therefore, applying J-aggregation for biological purposes remains a challenging issue.

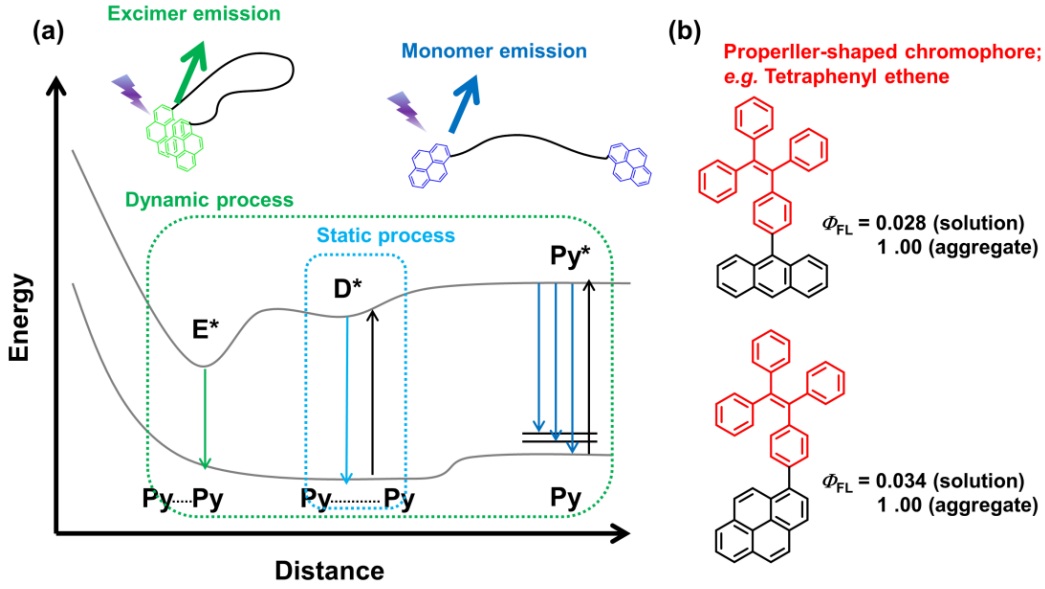

Figure 10. (a) Energy level diagram for excimer formation in pyrene. (b) Examples of AIEE active molecules.

4.2.5. Other

Photoswitchable fluorescence molecules have attracted considerable interest owing to their potential application for STORM. Typical examples include photochromic molecules as

shown in Figure 11a.[36] Molecules that undergo structural change in the presence of specific biochemical species to become fluorescent are also important. For example, Glu-HMRG (Figure 11b), developed by Urano, is used for the detection of small cancers, even those smaller than 1 mm across.[37]

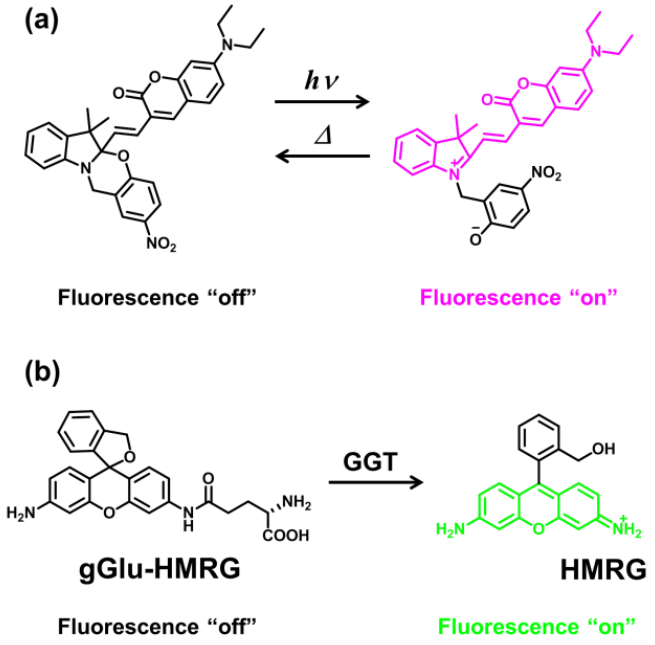

Figure 11. (a) A photoswitchable fluorophore based on photochromism. (b) Glu-HRMG, a fluorescent probe for the detection of cancer. GGT stands for γ-glutamyl transpeptidase.

4.3. Two-photon-induced fluorescent dyes

As previously mentioned, it is possible to excite two-photon active molecules exclusively at the focal point of the excitation laser light. Therefore, spatially high-resolution imaging can be performed through combination with TPFM. The design concepts for developing dyes possessing two-photon absorption cross sections ($\sigma^{(2)}$) have been theoretically and experimentally demonstrated (see section 5).[38] For example, molecules possessing long, highly planar π-conjugation systems, centrosymmetric structures with electron-donor and/or electron-acceptor moieties, *e.g.*, D-π-A-π-D or A-π-D-π-A, structures with strong one-photon absorptions, molecules with lower detune energies, and those with narrow one- and two-photon absorption bands are potentially desirable two-photon absorbers. Species showing a super-large $\sigma^{(2)}$ of over 10,000, and, although rarely, even over 100,000, have been found, particularly oligothiophene macrocycles[39] and squaraine derivatives.[40] However, for TPFM imaging, the two-photon action cross-section $\Phi\sigma^{(2)}$ of the fluorophore, the multiple of

fluorescence quantum yield and the two-photon absorption cross section, *i.e.*, the brightness when using TP excitation, are more practical parameters. In addition, excitation wavelength and fluorescence wavelength, photostability, fluorescence solvatochromic response, and fluorophore size should be considered. For example, fluorophores with an extremely large $\sigma^{(2)}$, such as squaraine derivatives, are usually non-fluorescent or too large in molecular size, and therefore not appropriate for bio-imaging. In addition, a D-π-A form dipolar molecule can exhibit fluorescence solvatochromism, but its value of $\sigma^{(2)}$ can be much lower than that of the D-π-A-π-D or A-π-D-π-A form quadrupolar molecules. Furthermore, the fluorophores discussed in section 4.1 should have high photostability and red-shifted absorption and fluorescence, as we see with cyanine 5, but often exhibit lower $\Phi\sigma^{(2)}$ than a dipolar molecule. Some examples of important two-photon active fluorophores are shown in Figure 12.

Currently, one of the most serious drawbacks of TPFM is its high cost, as noted above. One potential solution to this problem is the introduction of the fiber laser, which is easy to handle, cheap, and small. For bio-imaging, a fiber laser that can oscillate at 1050 nm, *i.e.*, within the biological window, is desired. However, a fluorophore compatible with such a laser source has not yet been developed.

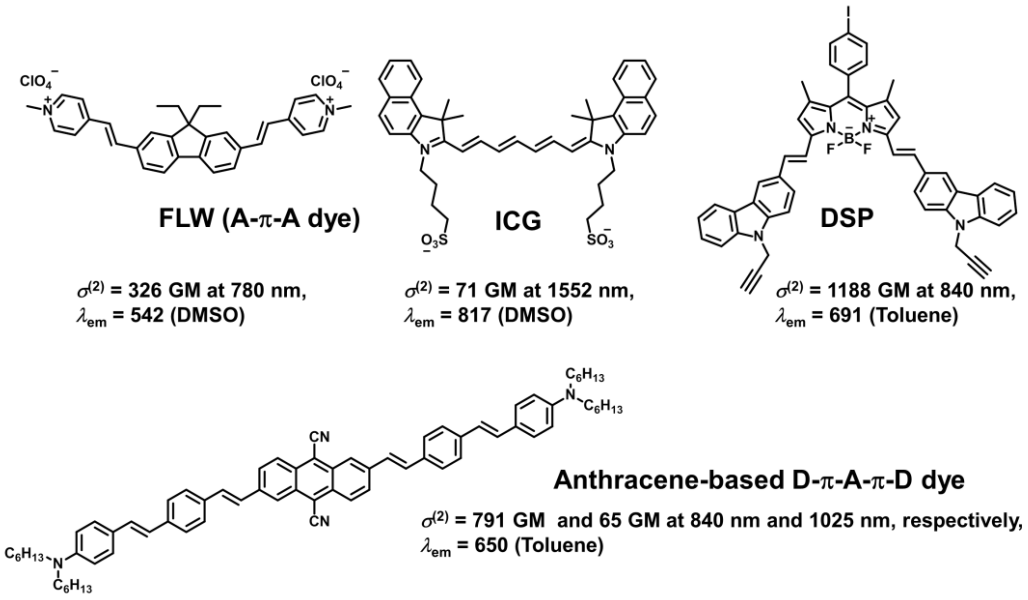

Figure 12. Chemical structures of two-photon active fluorophores. The data for FLW, ICG, DSP, and the anthracene-based D-p-A-p-D dye were taken from references [41], [42], [43], and [44], respectively.

4.4. Overview

As has been mentioned repeatedly, both the research purpose and instrument availability should be considered when selecting and developing new fluorescent probes, because these issues determine the required fluorescence properties.

The above-described fluorophores were roughly divided into three types: 1) fluorophores possessing high brightness and photostability (section 4.1.), 2) fluorophores capable of qualitative analysis (section 4.2.1. - 4.2.4.), and 3) two-photon active fluorophores (section 4.3.). In most cases, there is a trade-off between the characteristics of these three fluorophore types. Several exceptions, *e.g.*, the nature of the fluorophores shown in section 4.2.4., like AIEE, were ignored here because such functions are not derived from the inherent electronic nature of the fluorophores and can be applied to the molecules in section 4.1. or 4.3. through synthetic organic methods.

It is most important here to consider what fluorescence properties are in demand for fluorophores. The author considers that the highly bright and photostable fluorophores mentioned in section 4.1. are currently well represented compared to the fluorophores in section 4.2., as can be inferred from the fact that a lot of their derivatives are commercially available. Additionally, in the application of the bright fluorophores in section 4.1. for single-molecule imaging, environmental responsiveness is often an unwanted property owing to the principle of the instruments. Instead, it is much better to increase the brightness and photostability of the fluorophores in section 4.2., especially the fluorescence solvatochromic dyes like ESICT- and/or ESIPT-based dyes, *i.e.*, the fluorophores in section 4.2.2., because the lower brightness and photostability of the dyes often lead to significant drawbacks. For example, it may be required to use an excess of the dyes to continue prolonged imaging, which can change the properties of the samples of interest. Moreover, it is also possible that degradation of dyes occurs with the generation of reactive species which may damage the samples. The reason why particularly the fluorophores in section 4.2.2. should be modified is that the fluorophores in section 4.2.1. no longer have scope for further design, and the fluorophores in section 4.2.3. are similar in structure to the ESICT dyes. Finally, with respect to two-photon active dyes in section 4.3., the development of fluorophores compatible with fiber lasers may be the most urgent issue, since it addresses the crucial drawbacks of TPFM.

5. Key photophysical processes and rules in this thesis

Intersystem crossing promoted by El-Sayed's rule:[45] Intersystem crossing is a non-radiative process in which the zero-vibronic level of one excited state is equi-energetically transferred to the high vibronic level of an adjacent excited state with a different spin-multiplicity, usually involving the transition from singlet to triplet. Through this process, the dye becomes less fluorescent and occasionally shows phosphorescence. When the wave function for the transition between electronic states is solved using a roughly approximated Hamiltonian, the transition possibility between single and triplet states is calculated as zero. However, using a perturbed Hamiltonian demonstrates that singlet and triplet states contain each other's natures, a phenomenon known as mixing. El-Sayed further evaluated the mixing by using spin-orbital interactions, and then demonstrated that the mixing between π–π^* transitions and between n–π^* transitions is weak, while the mixing between π–π^* and n–π^* transitions is strong. This has become known as El-Sayed's rule, and explains why many carbonyl compounds are less fluorescent owing to their $S_1(n$–$\pi^*)$ states.

Internal conversion promoted by the energy gap rule:[46] Internal conversion is a non-radiative process in which the zero-vibronic level of one excited state is equi-energetically transferred to the high vibronic level of a lower excited state with the same spin-multiplicity, usually indicating the transition from singlet to singlet. The following vibronic relaxation process leads the dye to its ground state. The energy gap rule indicates that the internal conversion rate constant becomes smaller with an increase in the energy gap between ground and excited state. This can be explained as follows:

First, assume that internal conversion occurs at the cross points of energy potential surfaces of state 1 (before transition) and state 2 (after transition). If the vibronic quantum number of state 2 is small, *i.e.*, the energy gap between states 1 and 2 is small, the Frank-Condon factor, which is contained in the wave function with respect to the electronic transition, around the cross points of energy potential surfaces becomes large. Therefore, the transition rate constant from state 1 to 2 also becomes large. This principle is applicable to the intersystem crossing rate constant.

Typical solvatochromic fluorophores tend to show largely red-shifted fluorescence and large Stokes shifts in highly polar solvents, along with a decrease in fluorescence quantum yields. In most cases, this trend can be explained by the energy gap rule, and it is known that the internal conversion rate constant exponentially increases with an increase in fluorescence wavelengths. Furthermore, this rule also explains why the fluorescence quantum yield of most

dyes exhibiting fluorescence around near-infrared regions tends to be low.

Fluorescence solvatochromism:[47] Typically, a fluorophore possesses a larger dipole moment in the excited state (μ_e) than in the ground state (μ_g). Following excitation, the solvent dipoles re-orient or relax around μ_e, which lowers the energy of the excited state, thus leading to a process known as solvent relaxation. This effect becomes larger with an increase in solvent polarity, resulting in emission at lower energies and longer wavelengths. The shift of the emission band in response to change in solvent polarity is known as fluorescence solvatochromism. In general, only fluorophores with large dipole moments in the ground or excited state, *e.g.*, a dye possessing a D-π-A structure, display large fluorescence solvatochromism. In contrast, apolar molecules such as unsubstituted aromatic hydrocarbons or symmetric molecules such as fluorescein, rhodamine, and cyanine are much less solvatochromic. In addition, ionic compounds often possess a larger μ_g than μ_e, and display "negative" fluorescence solvatochromism, typified by Reichardt's dye.[48]

Two-photon absorption: Two-photon absorption is a third-order, non-linear optical phenomenon in which one dye absorbs two photons simultaneously. This phenomenon was predicted by Göppert-Mayer in the 1930s but only experimentally demonstrated after the invention of the laser light source. The most important difference between one-photon and two-photon absorption is that two-photon absorption is proportional to the square of the intensity of the excitation light, whereas one-photon absorption occurs linearly. Therefore, the two-photon active dyes can be excited exclusively at the focal point of the laser light, especially a pulse laser, which generates strong photon density. This characteristic is applicable to a variety applications such as microscopy, microfabrication, 3D data storage, photodynamic therapy, optical power limiting, up-converted lasing, and localized release of bio-active species.

The theoretical equation concerning two-photon absorption cross sections is very clearly explained in the reference [38] by Anderson *et al*. Therefore, I mainly cite the sentences in that paper and describe the equation as follows:

If the light is plane-polarized, the value of $\sigma^{(2)}$ for a transition from ground state *g* to final state *f* at the maximum of a two-photon band with a Lorentzian line shape is given by Equation (1).

$$\sigma^{(2)} = \frac{2\pi h v^2 L^4}{\varepsilon_0 n^2 c^2}\left(\frac{1}{\Gamma}\right) S_{fg}$$

$$\text{where } S_{fg} = \left[\sum_i \frac{\langle \boldsymbol{\mu}_{gi}\boldsymbol{\mu}_{if}\rangle}{(E_{gi} - hv)}\right]^2 \qquad (1)$$

Here, E_{gi} is the energy gap between the ground state and an intermediate state i, and Γ is the half-width at half-maximum (HWHM) of the two-photon band in energy units. The summation is over all the states of the molecule. The factor $L = (n^2+2)/3$, where n is the refractive index, represents the enhancement of the optical field in the medium relative to that in a vacuum. The term $\boldsymbol{\mu}_{kl}$ is the amplitude of the transition dipole moment induced by the electric field of a light wave whose frequency is in resonance with the energy difference between the k and l states. The various transition dipole moment vectors $\boldsymbol{\mu}_{kl}$ rotate with the molecule in solution, therefore, an average must be calculated of their projection onto the direction of the optical field, over all orientations of the molecule. This is the origin of the term in pointed brackets in Equation (1). Derivation of this average is not straightforward, but the result is 1/5 if all moments $\boldsymbol{\mu}_{kl}$ and $\boldsymbol{\mu}_{if}$ are co-parallel. This is true of the great majority of strong two photon absorbers, therefore we may omit the vector notation to give, after some rearrangement, Equation (2),

$$S_{fg} = \frac{1}{5}\left[\left(\frac{\Delta\mu_{gf}\mu_{gf}}{hv}\right)^2 + \sum_{i\neq g,f}\left(\frac{{\mu^2}_{gi}{\mu^2}_{if}}{\left(E_{gi} - hv\right)^2}\right)\right] \qquad (2)$$

where $\Delta\mu_{gf}$ is the change in the static dipole moment in the final state relative to the ground state. The two parts of Equation (2) have been described as the "dipolar" D term
and "two-photon" T term, respectively. If a centrosymmetric molecule is used, the D term becomes zero, and the $\sigma^{(2)}$ value can be described by Equation (3).

$$\sigma^{(2)} \approx C\frac{{\mu^2}_{gi}{\mu^2}_{if}}{\left(\left(E_{gi}/hv\right) - 1\right)^2 \Gamma} \qquad (3)$$

The essence of these equations focuses on the molecular design concept as mentioned in section 4.3.

Photostability: The photostability of fluorophores has been discussed along with the use of fluorescence microscopy. However, a systematic description of the phenomenon was not performed until recently. According to Branchard (2014),[17] there are three main reasons for photobleaching and photoblinking of fluorophores:

1) In general, a triplet state possesses a longer lifetime, usually in the order of a microsecond,

than that of a singlet state, which is usually in the order of a nanosecond for fluorescent molecules. Therefore, an electron-transfer reaction that produces non-fluorescent radical species and dye degradation might be triggered by the generation of the fluorophore triplet state. Such redox reactions would also occur in the presence of impurities, molecular oxygen, or compartments in biological systems. If electron transfer to oxygen occurs, a superoxide radical is generated. Additionally, energy transfer to oxygen is also possible, resulting in singlet oxygen, a strong oxidation agent. Both species are sufficiently reactive to cause photobleaching and photoblinking, and destroy biochemical species in the system.

2) In general, organic dyes immediately generate a first excited state after photoexcitation, according to Kasha's rule. However, when additional higher excited states, *i.e.*, S_n and T_n, where $n > 1$, are generated through sequential one-photon absorption or simultaneous multiphoton absorption via strong illumination with a pulse laser, such high energetic states may cause photoreactions or bond cleavage.

3) Polymethine structures often undergo *cis-trans* isomerization. As demonstrated by stilbene, this isomerization may generate non-fluorescent species.

6. Functional fluorescent molecules with electron donors and/or acceptors

Dipolar D-π-A (push-pull) molecules: Prodan, as discussed above, is a typical D-π-A molecule. The fluorophores based on this structure can exhibit fluorescence solvatochromism, and show two-photon activity. Therefore, the D-π-A characteristic is an important molecular design concept to add such functions to a chromophore. However, fluorescence quantum yield of D-π-A fluorophores tend to be small in either non-polar or much polar media, or both. In most cases, the quenching in apolar media is due to intersystem crossing based on El-Sayed's rule, while the quenching in polar media is due to internal conversion based on the energy gap rule. In addition, D-π-A fluorophores have the disadvantages of much lower brightness and photostability than those fluorophores shown in section 4.1.

Quadrupolar D-π-D, A-π-A, D-π-A-π-D, and A-π-D-π-A molecules: Although these dyes do not show strong solvatochromism, they can exhibit large two-photon cross sections. D-π-A-π-D structures tend to possess larger two-photon cross sections than do A-π-D-π-A structures. Considering that two-photon active dyes for TPFM are still rare, these molecular design concepts are also important.

7. Philosophy of this thesis

In sections 1–4, the applications of fluorophores in biomedical fields, as well as the characteristics and advantages/disadvantages of the existing optical techniques and the existing fluorophores, were described in detail. It was emphasized that the choice and development of fluorophores should take into account the available instruments and purpose of the research. As a result, in section 4.4. the author concluded that the development of 1) solvatochromic fluorophores with high brightness, photostability, and red-shifted absorption/fluorescence and 2) two-photon active fluorophores compatible with 1050 nm fiber lasers is imperative.

7.1. Purpose of this thesis

The aim of this thesis is to contribute to the development of the above-mentioned solvatochromic and two-photon active fluorophores, which are not represented by existing molecules. In achieving this goal, the author focused on pyrene as a key chromophore and performed the following: 1) the development of novel pyrene-based fluorophores possessing multiple electron-donor and/or electron-acceptor moieties, and 2) the study of the structure-property relationships of such pyrene fluorophores.

7.1.1. What is pyrene?

Characteristics: Pyrene is an aromatic hydrocarbon with structural features such as high rigidity, planarity, and D_{2h} symmetry. The 1-, 3-, 6-, and 8-positions of pyrene are highly reactive. Accordingly, it is possible to produce novel pyrene derivatives while maintaining a certain level of symmetry in the molecule, *e.g.*, 1,3,6,8-tetraphenylpyrene, which possesses D_2 symmetry. Pyrene and its derivatives tend to show higher extinction coefficients than other aromatic hydrocarbons such as naphthalene, fluorene, anthracene, and perylene (Figure 13).[49] Additionally, the long singlet lifetime (> 100 ns) and highly structured fluorescence spectrum of pyrene, *i.e.*, the Ham effect, due to forbidden S_0-S_1 (HOMO-1–LUMO and HOMO-LUMO+1) transitions are well known. However, these characteristics are easily lost through slight modifications to the parent pyrene, even mono-alkylation. Therefore, they are of little significance in terms of future research on pyrene derivatization.

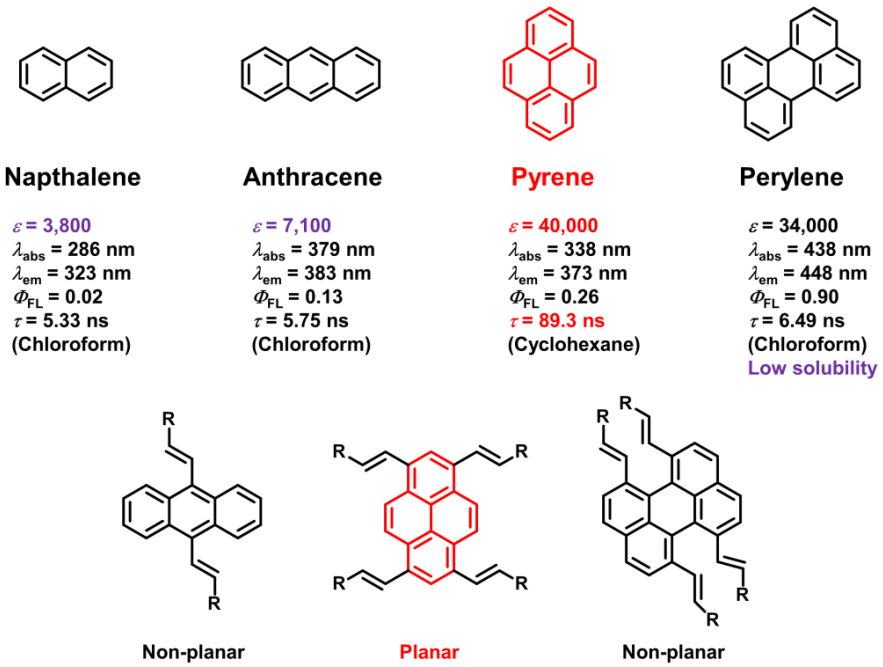

Figure 13. Chemical structures of polycyclic aromatic hydrocarbons. The data were taken from reference [49].

Applications: Historically, pyrene has been used as a fluorescent probe by utilizing the Ham effect and its ability to form excimers, as discussed in section 4. Pyrene is often used as a building block for electronic devices[50] such as OLEDs, organic field effect transistors (OFETs), and for the construction of carbon materials[51] such as carbon nanotubes (CNTs) or graphene. The hydrophobicity of pyrene is also important for its use as a compatibilizing agent for CNTs[52] and its distribution in the hydrophobic areas of plasma membranes, *i.e.*, the lipid rafts discussed in section 2.[3]

Derivatization: In most cases, a parent pyrene is derivatized by the introduction of functional groups to the 1-, 6-, 3-, or 8-positions (Figure 14). The majority of modifications are performed using phenyl, vinyl, and ethynyl groups to extend the π-conjugation system of pyrene. Müllen has previously established a synthetic protocol for such modification.[51] It is noteworthy that, ***almost all pyrene derivatives modified by more than two substituents possess both structural and electronic symmetry.*** Substitution on the 2- or 7-positions is often performed with tertiary alkyl groups. This is usually performed to enhance the solubility of the dyes or to facilitate the modification of 1,3-positions because it is known that π-extension at 2- or 7-position gives much less perturbation to pyrene compared to 1-, 6-, 3-, or 8-positions.[53] Recently, the 4-, 5-, 9-, and 10-positions, which are usually much less reactive, have been functionalized. However, the observed substitution effects on the

photophysical and electrochemical properties were not significant.[54] In fact, this synthetic approach was mainly developed for the construction of carbon materials discussed above.

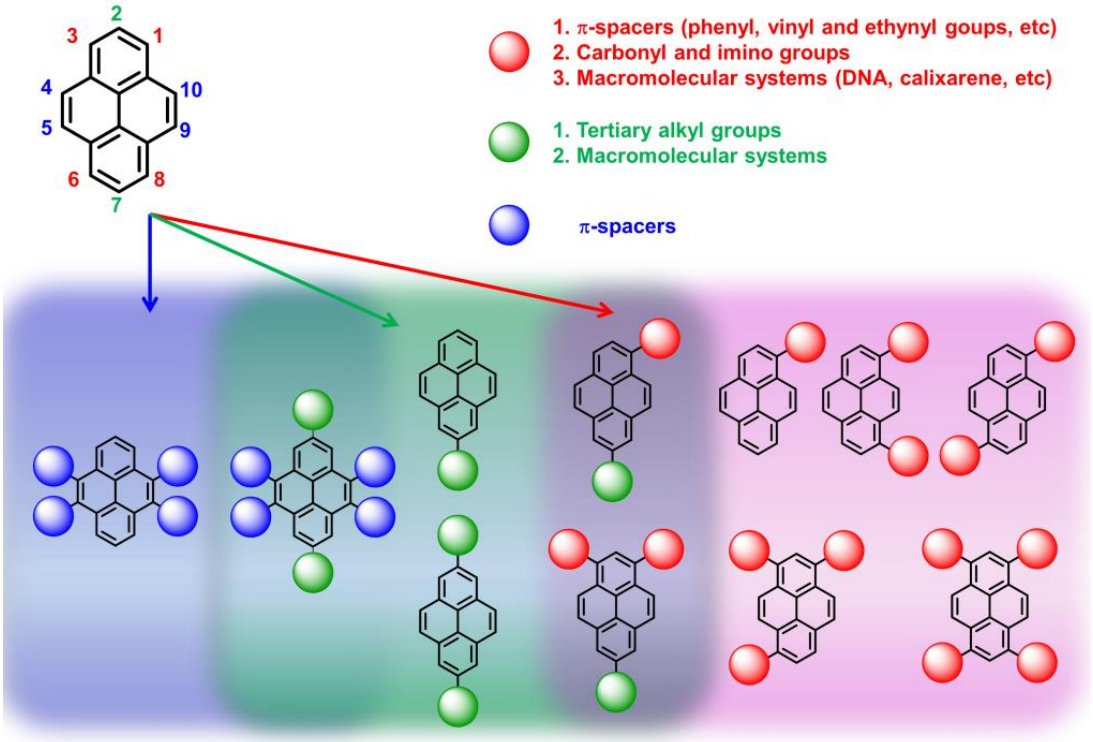

Figure 14. Derivatization schematic of the pyrene chromophore.

7.1.2. Why Pyrene?

Compared with other aromatic hydrocarbons, pyrene has several advantages, as described below:

Structural advantages: Pyrene has four main reactive points, *i.e.*, the 1-, 3-, 6-, and 8-positions. Therefore, its modification is flexible for achieving specific properties, as illustrated in Figure 13. Unlike fluorene, pyrene can be derivatized and still maintain high symmetry, which is advantageous for producing efficient two-photon absorption species. Furthermore, anthracene and perylene possess reactive points at the 9-, 10- and 1-, 6-, 7-, 12-(bay-area) positions, respectively (Figure 13). In contrast with pyrene, the modification of anthracene and perylene at such positions often leads to dyes with a twisted π-system due to inherent steric hindrance, *e.g.*, that seen for the hydrogen at the peri-position in anthracene. This may be inappropriate for both linear and non-linear photophysical properties. Finally, pyrene and its derivatives have a better solubility than lager aromatic hydrocarbons such as perylene. This feature is also important in facilitating derivatization.

Electronic structural advantages: Pyrene has more stable π–π* excitation energy than

naphthalene and fluorene due to its larger π-system. Therefore, if each chromophore possesses the same structure, *e.g.*, D-π-A, pyrene derivatives should exhibit absorption and fluorescence at longer wavelengths. In addition, when assuming that each chromophore is substituted by a carbonyl group like a formyl group, the El-Sayed-type intersystem crossing of pyrene dyes should be most inefficient because the energy gap between $S_1(\pi-\pi^*)$ and $S_2(n-\pi^*)$ of pyrene would be the largest. Finally, pyrene has more π-electrons than naphthalene and fluorene. This feature is advantageous for an increase in the two-photon absorption cross-section.

7.1.3. Why functionalize with electron donors and/or acceptors?

The importance of dipolar, *i.e.*, D-π-A push-pull, and quadrupolar, *i.e.*, D-π-D, A-π-A, D-π-A-π-D, and A-π-D-π-A, structures, realized by electron-donor and/or electron-acceptor moieties, were discussed in section 6. Briefly, dipolar and quadrupolar structures are useful motifs to provide the fluorophores with ESICT-based fluorescence solvatochromism and two-photon activity, respectively. As mentioned above, in most cases, pyrene has been previously modified at the 1-, 3-, 6-, and 8-positions with conservation of structural and electronic symmetry. Thus, the number of pyrene fluorophores possessing more than two electronic donor and/or acceptor moieties is limited. This fact is very surprising, because other chromophores such as naphthalene, fluorene, and anthracene have been well derivatized in such a manner. To the best of the author's knowledge, there have only been three examples of modified pyrenes with the clear purpose of changing its photophysical properties due to dipolar and quadrupolar structures (Figure 15). One is the development of pyrene-based D-π-A dyes by Saito (2007)[55] and second is the preparation of D-π-D dyes by Cho (2008).[56] However, the former work lacked a detailed discussion of the fluorescence properties, photostability, and fluorescence mechanism, *i.e.*, the structure-property relationship. Although the latter demonstrated that pyrene is a promising building block as a two-photon absorber, the fluorophores in the work were not developed with clearly defined requirements, therefore, it is possible to find other examples with similar properties from existing fluorophores. At third, while Müllen and his group developed dipolar and quadrupolar pyrene derivatives, they did not measure all their fluorescence properties.[54]

From these viewpoints, investigations into electron-donor and/or electron-acceptor functionalized pyrene fluorophores have a large scope of work to be undertaken.

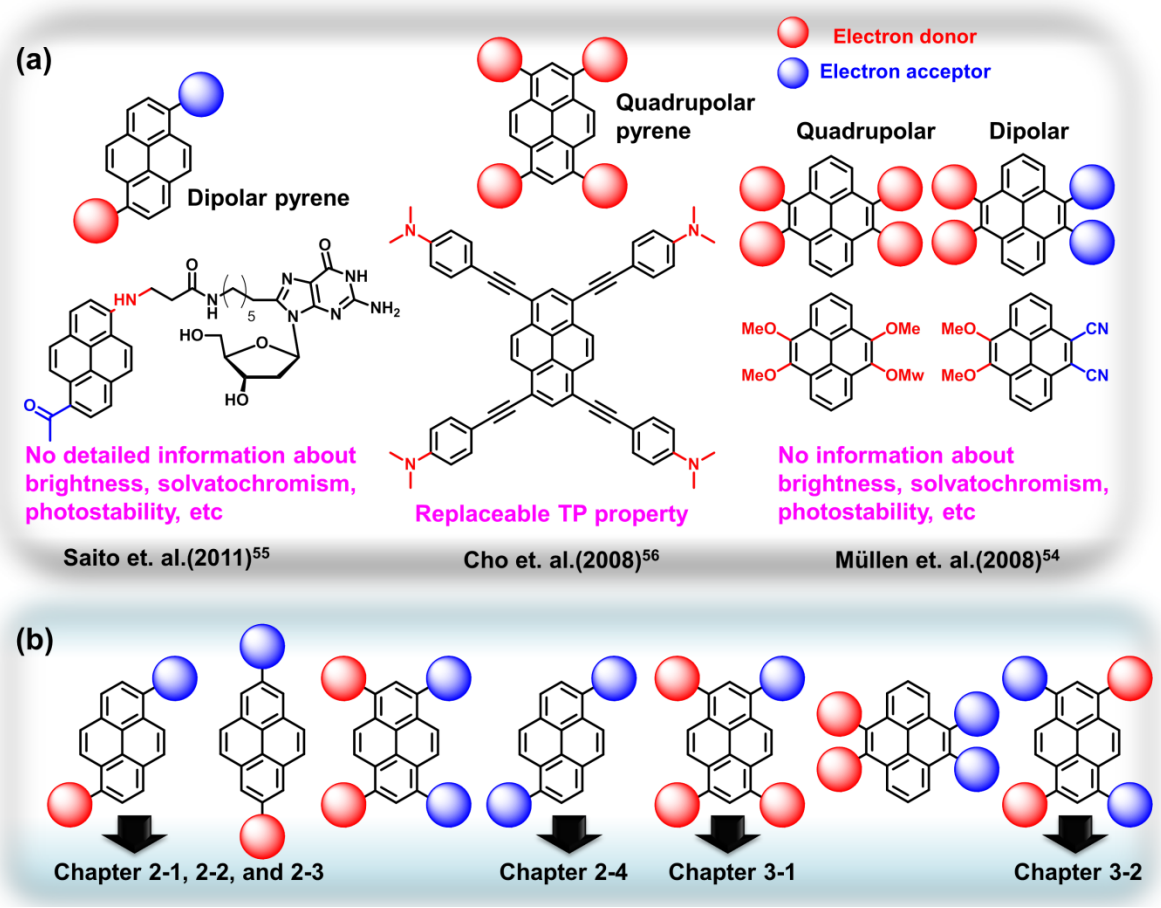

Figure 15. (a) Existing functional pyrene fluorophores with electron-donor and/or electron-acceptor moieties. (b) Examples of the structures to be explored.

7.2. Overview

In this thesis, several pyrene fluorophores are developed. Some of their unique and useful properties were then investigated, as described below:

In Chapter 1, the author demonstrated that carbonyl groups, which are typical electron acceptors, changed the photophysical properties of pyrene in various ways depending on the kind of carbonyl group employed, *e.g.*, aldehyde, carboxylate, and so on. In addition, the effects specific to pyrene were also investigated. Next, it was revealed that alkyl groups, which are usually not used for tuning photophysical properties, clearly work as electron donors and enhance the fluorescence quantum yield of pyrene.

In Chapter 2, several functional pyrene derivatives with electron donors and/or acceptors were developed. The author successfully developed pyrene-based D-π-A fluorophores showing a much higher brightness, photostability, red-shifted absorption and fluorescence than Prodan. Furthermore, the D-π-A fluorophores exhibiting a high quantum yield in water, or a high sensitivity to H-bond donor media, were also developed. Finally, a

pyrene-based A-π-A fluorophore that can exhibit a large two-photon absorption cross-section around 1000 nm, red fluorescence, and high quantum yield, was obtained.

In Chapter 3, further advanced pyrene derivatives with electron donors and/or acceptors were developed. The author developed pyrene-based D-π-A fluorophores that exhibit a similar fluorescence wavelength to Nile Red, *i.e.*, strongly red-shifted fluorescence, with enhanced photostability. In addition, a pyrene-based A-π-D-π-A fluorophore that exhibited a larger two-photon absorption cross-section around 1050 nm than the above-mentioned A-π-A fluorophores, with fluoresce in the near-infrared region, was developed. All of the fluorophores developed in chapters 2 and 3 possess novel properties compared with existing fluorophores.

In Chapter 4, the author attempted the imaging of a model plasma membrane using pyrene-based D-π-A fluorophores. It was demonstrated that pyrene fluorophores could be a promising tool for lipid raft imaging. Next, the author developed a novel supramolecular probe that only fluoresces in living cells, and could therefore facilitate background-free imaging. Although the fluorophore used in this study was Nile Red and not pyrene, all fluorophores can potentially be used for this application. In this regard, this research could be very important in paving the way for all synthetic fluorophores.

Finally, the remaining drawbacks of pyrene should be addressed herein. Pyrene has a larger structure compared to naphthalene and fluorene. Therefore, it cannot mimic natural fluorescent molecules like tryptophan. Additionally, the increase in the transition moment due to the π-extension of pyrene and other polycyclic aromatic hydrocarbons is much lower than that for polymethine-based molecules and rod-like structured molecules. In fact, it was very difficult to produce a super-bright pyrene analogue. If polymethine structures were introduced into the pyrene core, the obtained derivatives may be brighter, as can be seen with the pyrene-based A-π-A fluorophore in Chapter 2. However, such compounds would be inappropriate for the construction of D-π-A fluorophores. It can be speculated that the fluorophores become much less fluorescent because of the large charge separation in the excited state. Such trade-offs could be the largest challenge to be overcome when using pyrenes as fluorophores in future studies.

8. Abstracts for this thesis

Chapter 1. Substitution Effects of Carbonyl and Alkyl Groups on Photophysical Properties of Pyrene Chromophore

In this chapter, the author re-investigated the substitution effects of carbonyl groups (typical electron acceptors), and alkyl groups (unusual electron donors), on the photophysical properties of pyrene. This was achieved by absolute measurements of fluorescence quantum yields, recently improved density functional theory (DFT), and/or electrochemical methods.

In the first section, it was demonstrated that formyl and acetyl groups lead to much less fluorescent pyrene derivatives, with $\Phi_{FL} < 0.01$ in most solvents, due to not only El-Sayed-type intersystem crossing, but also internal conversion promoted by proximity effects. In contrast, carboxylic acid or ester groups make pyrene derivatives more fluorescent, with $\Phi_{FL} > 0.50$ in most solvents. Furthermore, the author has previously demonstrated that the fluorescence intensities of pyrene derivatives substituted by *N*-alkyl or *N*,*N*-dialkylcarboxamides are dependent on the solvent's viscosity due to twisting structures between pyrene and carbonyl groups. These various effects derived from carbonyl group substitution were characterized by DFT calculations and are discussed in this section.

In the second section, it was demonstrated that primary, secondary, and tertiary alkyl groups could work as electron donors through conjugation between the π-system of pyrene and the σ-orbital of the C-H or C-C bonds of the alkyl groups, i.e., σ–π conjugation. This effect increases the HOMO levels of pyrene and reduces the HOMO-LUMO gap. Therefore, the HOMO-LUMO transition of alkylated pyrene corresponds to the S_0-S_1 transition, though the transition corresponds to the S_0-S_2 transition for the parent pyrene. Accordingly, the oscillator strengths of the S_0-S_1 transition in alkylated pyrene increases, even though the transition is forbidden in the parent pyrene. As a consequence, alkylated pyrenes exhibit fluorescence at longer wavelengths with a high fluorescence quantum yield depending on the number of alkyl groups, *e.g.*, the Φ_{FL} values for pyrene and 1,3,6,8-tetra-*n*-butyl pyrene in methanol are 0.17 and 0.68, respectively. These results show that alkyl groups, usually used for the enhancement of the solubility of rigid compounds, might be useful to tune the photophysical properties of chromophores.

Chapter 2. Synthesis and Photophysical Properties of Pyrene-based D-π-A and A-π-A Fluorophores

In this chapter, the author developed three types of pyrene-based D-π-A fluorophores, as well as an A-π-A fluorophore, and investigated their photophysical properties.

In the first section, the author synthesized pyrene-based D-π-A fluorophores where the electron donors and acceptors were alkyl groups and formyl groups, respectively. The most interesting feature of these fluorophores is that they show sensitivity to not only the polarity but also to the H-bond donor ability of each solvent. For example, 3,6,8-tributylpyrene-1-carbaldehyde gives λ_{em} values of 437 nm, 460 nm, 458 nm, and 484 nm, and Φ_{FL} values of 0.06, 0.80, 0.37, and 0.80 in THF, chloroform, DMSO, and ethanol, respectively. The introduction of alkyl donors into 1-formylpyrene increases the HOMO levels, leading to a large energy gap between $S_1(\pi-\pi*)$ and $S_2(n-\pi*)$. Therefore, the energetic inversion and mixing of these two states, which induce intersystem crossing and internal conversion, respectively, are easily prevented by H-bond donor solvents that destabilize the $n-\pi*$ excited state. If chloroform, an H-bond donor, is used, the obtained fluorophore showed stronger emissions than in H-bond acceptor solvents, and even more so in polar solvents such as DMSO. This characteristic is very interesting because D-π-A fluorophores cannot usually distinguish between two solvent effects, *i.e.*, polarization and/or H-bonding interactions.

Next, the author developed pyrene analogs of Prodan, *i.e.*, D-π-A fluorophores where the electron donors and acceptors are dialkyl amino, and formyl or ketone groups, respectively. An interesting feature of these fluorophores is that they show higher extinction coefficients, with the ε values for pyrene dyes and Prodan being 25000 and 17000, respectively. They also display red-shifted absorption, compatible with commonly used 405 nm diode lasers, and red-shifted fluorescence solvatochromism, i.e., from sky-blue to orange, compared to Prodan. Furthermore, these fluorophores show very high fluorescence quantum yields (Φ_{FL} = 0.70 ~ 0.90) in both apolar (hexane) and polar (methanol) solvents, with high photostability. This is because the $n-\pi*$ excited state, which causes intersystem crossing, becomes energetically inefficient even in apolar solvents when using pyrene as the chromophore. Therefore, the nature of the $\pi-\pi*$ excited state becomes dominant. In addition, the changes in the dipole moment of the fluorophores between the ground and excited states are not too large. Therefore, internal conversion in highly polarized solvents is suppressed. These results indicate that pyrene is a good building block for the construction of D-π-A fluorophores. These fluorophores possess a high fluorescence quantum yield and have a high fluorescence

quantum yield, which is important in obtaining high photostability.

In the third section, the author synthesized pyrene-based D-π-A fluorophores where the electron donor and acceptor are dialkylamino, and *N*-alkyl or *N*,*N*-dialkylamide groups, respectively. By intentionally using very weak acceptors such as alkylamide groups, fluorophores that show a high fluorescence quantum yield (Φ_{FL} > 0.90) in apolar and polar solvents, and even in water, were achieved. This is because alkylamide groups do not generate an n–π* excited state around S_1 due to the high stabilization of the *n*-orbitals by inter-amide conjugation, even in apolar solvents. Furthermore, the changes in the dipole moment of the fluorophores between the ground and excited state are small even in polar solvents. As a result, intersystem crossing in apolar solvents, and internal conversion in polar solvents, are inefficient. Interestingly, this is the first example of solvatochromic fluorophores showing high brightness in both apolar solvents and water. However, the solvatochromic responsiveness of the fluorophores is not strong compared to existing D-π-A fluorophores.

Finally, the author synthesized a pyrene-based A-π-A fluorophore where a pyridinium moiety was used as an electron acceptor, connected to pyrene through vinyl groups as a spacer. Surprisingly, this fluorophore exhibits a large two-photon absorption cross-section around 1000 nm ($\sigma^{(2)}$ = 1100 GM and 380 GM at 950 nm and 1050 nm, respectively). This indicates that it could be compatible with a 1050 nm fiber laser. Furthermore, it shows fluorescence around 650 nm with a high fluorescence quantum yield (Φ_{FL} = 0.80 in DMSO). Therefore, this fluorophore could be efficiently excited and fluoresce in the biological window. In fact, by using TPFM and this fluorophore, living mitochondrial imaging was facilitated with a much lower excitation laser power compared to commercially available mitochondrial probes. These features have not been found in other existing two-photon active fluorophores. The properties discussed above are largely derived from the structural and electronic characteristics of pyrene, such as its high planarity, symmetry, rigidity, and number of π-electrons. This indicated that pyrene-based quadrupolar structures are promising for the construction of two-photon active fluorophores compatible with fiber lasers.

Chapter 3. Synthesis and Photophysical Properties of Pyrene-based Advanced Dipolar and Quadrupolar Fluorophores

In this chapter, the author attempted to improve the photophysical properties of the pyrene-based D-π-A fluorophores and synthesize novel pyrene-based A-π-D-π-A fluorophores.

A new pyrene-based D-π-A fluorophore that possessed three dialkylamino groups as electron donors was synthesized. This fluorophore showed red-shifted absorption to around 480 nm, and fluorescence shifted from green to red, with a high fluorescence quantum yield (Φ_{FL} = 0.50~0.90) in apolar and polar solvents. This red-shifted fluorescence was similar to Nile Red, which is known as an exceptional solvatochromic fluorophore that emits at longer wavelengths. Although the absorption coefficient of the pyrene fluorophore was almost half that of Nile Red, its photostability was much higher. Therefore, this new pyrene-based D-π-A fluorophore could be extremely useful for practical applications.

In the second section, the author synthesized a pyrene-based A-π-D-π-A fluorophore where two alkoxy groups are introduced to the A-π-A fluorophore shown in chapter 2. This fluorophore showed better two-photon absorption around 1000 nm ($\sigma^{(2)}$ = 1600 GM at 950 nm and 1050 nm, respectively) and fluorescence in the near-infrared region (λ_{em} = 726 and Φ_{FL} = 0.26 in DMSO). The fluorescence quantum yield became lower than that of the fluorophore the author developed in chapter 3, with the fluorescence wavelengths being located entirely in the biological window (650-1100 nm). Notably, this is the first example of the development of pyrene dyes possessing quadrupolar donor-acceptor systems. Therefore, the synthetic methodologies and structure-property relationships shown in this work should be valuable for future molecular design.

Chapter 4. D-π-A Fluorophores-based Imaging of Lipid Rafts and Development of Novel Supramolecular Probe

In this chapter, the author employed pyrene-based D-π-A fluorophores for practical applications, *e.g.*, the imaging of lipid rafts in plasma membranes. In addition, a supramolecular probe based on a fluorescent surfactant was developed as a new application for organic fluorophores, including D-π-A fluorophores.

In the first section, the application of pyrene-based D-π-A fluorophores as lipid raft probes was investigated. The fluorophore was introduced to several model membranes to investigate the effect of the compositions and the phase states of lipid vesicles on fluorescence, and the results were compared with Laurdan, a Prodan analog. The most significant result was that the pyrene fluorophores exhibited a much higher photostability in vesicles than Laurdan while maintaining the dependency of fluorescence on the compositions and phases of the vesicles. This is because a crucial drawback of existing solvatochromic fluorescent probes for lipid rafts is their limited photostability. Therefore, pyrene-based D-π-A fluorophores should be promising probes for prolonged visualization of lipid rafts. The appropriate structural modifications could also facilitate the localization of probes on the outer leaflet of plasma membranes in living cells.

In the second section, novel fluorescent polymerized micelles, comprising of a fluorescent detergent and cross-linker, that can be cleaved by reductive stimulus were developed. The micelles are small (~20 nm), stable in organic solvents, and much less fluorescent, *i.e.*, "off," due to H-aggregation of fluorophores inside the micelles. However, they become highly fluorescent, *i.e.* "on," only inside living cells, because of disruption of the micelles triggered by inherent reductive agents in the cells. This feature is useful for background-free imaging. Furthermore, a variety of fluorophores, including the above-mentioned pyrene dyes, can be applied to these micelles. Therefore, these results could potentially expand the applications of nanomaterials.

9. References

1. Simon, K.; Ikonen, E. *Nature* **1997**, *387*, 569-572.
2. (a) Brown, D. A.; London, E. *J. Biol. Chem.* **2000**, *275*, 17221-17224. (b) Simons, K.; Toomre, D. *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 31-39. (c) Munro, S. *Cell* **2003**, *115*, 377-388. (d) Anderson, R. G. W.; Jacobson, K. *Science* **2002**, *296*, 1821-1825. (e) Dykstra, M.; Cherukuri, A.; Sohn, H. W.; Tzeng, S. J.; Pierce, S. K. *Annu. Rev. Immunol.* **2003**, *21*, 457-481. (f) Mukherjee, S.; Maxfield, F. R. *Annu. Rev. Cell Dev. Biol.* **2004**, *20*, 839-866. (g) Jacobson, K.; Mouritsen, O. G.; Anderson, R.G.W. *Nat. Cell Biol.* **2007**, *9*, 7-14. (h) Lingwood, D.; Simons, K. *Science* **2010**, *327*, 46-50.
3. Klymchenko, A. S.; Kreder, R. *Chemistry and Biology* **2014**, *21*, 97-113.
4. Kucherak, O. A.; Oncul, S.; Darwich, Z.; Yushchenko, D. A.; Arntz, Y.; Didier, P.; Mely, Y.; Klymchenko, A. S. *J. Am. Chem. Soc.* **2010**, *132*, 4907-4916.
5. (a) Keereweer, S.; Kerrebijn, J. D.; van Driel, P. B.; Xie, B.; Kaijzel, E. L.; Snoeks, T. J.; Que, I.; Hutteman, M.; van der Vorst, J. R.; Mieog, J. S.; Vahrmeijer, A. L.; van de Velde, C. J.; Baatenburg de Jong, R. J.; Lowik, C. W. *Mol. Imaging Biol.* **2011**, 13, 199-207. (b) Licha, K.; Olbrich, C. *Adv. Drug Delivery Rev.* **2005**, *57*, 1087-1108. (c) Rao, J.; Dragulescu-Andrasi, A.; Yao, H. *Curr. Opin. Biotechnol.* **2007**, 18, 17-25.
6. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy Third Edition*, Springer+Business Media, LLC; New York, **2006**, pp761-762.
7. Denk, W.; Strickler, J. H.; Webb, W. W. *Science*, **1990**, *248*, 73-76.
8. (a) Weissleder, R. *Nat. Biotechnol.* **2001**, *19*, 316-317. (b) Pu, Y.; Shi, L.; Pratavieira, S.; Alfano, R. R. *J. Appl. Phys.* **2013**, *114*, 153102.
9. Examples of 1050 nm femtosecond fiber laser; Ytterbius-1100 (Tekhnoscan), FCPA MicroJewel (IMRA), Origami (Onefive) or Fidelity (Coherent), and so on.
10. Kinjo, M. *Seikagaku* **2010**, *82*, 1103-1116.
11. Schermelleh, L.; Heintzmann, R.; Leonhardt, H. *J. Cell. Biol.* **2010**, *190*, 165-175.
12. Bastiaens, P. I. H.; Squire, A. *Trends Cell Biol.* **1999**, *9*, 48-52.
13. Campagnola, P. J.; Wei, M.-d.; Lewis, A.; Loew, L. M. *Biophys. J.* **1999**, *77*, 3341-3349.
14. (a) Kellogg, R. E.; Bennett, R. G. *J. Chem. Phys.* **1964**, *41*, 3042-3045. (b) Seybold, P. G.; Gouterman, M.; and Callis, *J. Photochem. Photobiol.* **1969**, *9*, 229-242. (c) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy Third Edition*, Springer+Business Media, LLC; New York, **2006**, pp637-639.
15. (a) Birge, R. R. "Kodak Laser Dyes," Kodak publication JJ-169 (**1987**). (b) Kubin, R. F.;

Fletcher, A. N. *J. Luminescence* **1982**, *27*, 455-462.

16. (a) Treibs, A.; Kreuzer, F. H. *Justus Liebigs Ann. Chem.* **1968**, *718*, 208-223. (b) Umezawa, K.; Citterio, Daniel, Suzuki, K. *Anal Sci* **2014**, *30*, 327-349.
17. Zheng, Q.; Juette, M. F.; Jockusch, S.; Wasserman, M. R.; Zhou, Z.; Altman, R. B.; Blanchard, S. C. *Chem. Soc. Rev.* **2014**, *43*, 1044-1056.
18. Diring, S.; Camerel, F.; Donnio, B.; Dintzer, T.; Toffanin, S.; Capelli, R.; Muccini, M.; Ziessel, R. *J. Am. Chem. Soc.* **2009**, *131*, 18177-18185.
19. Uchimura, M.; Watanabe, Y.; Araoka, F.; Watanabe, W.; Takezoe, H.; Konishi, G. *Adv. Mater.* **2010**, *22*, 4473-4478.
20. Moorthy, J. N.; Natarajan, P.; Venkatakrishnan, P.; Huang, D.-F.; Chow, T. J. *Org. Lett.* **2007**, *9*, 5215-5218.
21. (a) Kalyanasundaram, K.; Thomas, J. K. *J. Am. Chem. Soc.* **1977**, *99*, 2039-2044. (b) Niko, Y.; Kawauchi, S.; Otsu, S.; Tokumaru, K.; Konishi, G. *J. Org. Chem.* **2013**, *78*, 3196-3207. (c) Turro, N. J., Ramamurthyl, V. & Scaiano, J. C. *Modern Molecular Photochemistry of Organic Molecules* (University Science Books: Sausalito, California), **2010**, pp912.
22. (a) Kalyanasundaram, K.; Thomas, J. K. *J. Am. Chem. Soc.* **1977**, *81*, 2176-2180. (b) Niko, Y.; Hiroshige, Y.; Kawauchi, S.; Konishi, G. *Tetrahedron* **2012**, *68*, 6177-6185.
23. Sivaraman, G.; Anand, T.; Chellappa, D. *RSC Adv.* **2012**, *2*, 10605-10609.
24. (a) Wassam, W. A.; Lim, E. C. *J. Mol. Struct*. **1978**, *47*, 129-198. (b) Wassam, W. A.; Lim, E. C. *J. Chem. Phys.* **1978**, *68*, 433-454. (c) Wassam, W. A.; Lim, E. C. *J. Chem. Phys.* **1978**, *69*, 2176-2180.
25. Weber, G.; Farris, F. J. *Biochemistry*, **1979**, *18*, 3075-3078.
26. Kucherak, O. A.; Didier, P.; Mely, Y.; Klymchenko, A. S.; *J. Phys. Chem. Lett.* **2010**, *1*, 616-620.
27. Greenspan, P.; Mayer, E. P.; Fowler, S. D. *J. Cell Biol*.**1985**, *100*, 965-973.
28. (a) Weller, A. *Naturwissenschaften* **1955**, *42*, 175-176. (b) Weller, A. *Z. Elektrochem.* **1956**, *60*, 1144.
29. Frolov, Yu. L.; Sapozhnikov, Yu. M.; Barer, S. S.; Pogodaeva, N. N.; Tyukavkina, N. A. *Izu. Akad. Nauk. SSSR, Ser. Khim.* **1974**, 2364.
30. Loutfy, R. O.; Arnold, B. A. *J. Phys. Chem.* **1982**, *86*, 4205-4211.
31. Kim, H. M.; Jeong, B. H.; Hyon, J.-Y.; An, M. J.; Seo, M. S.; Hong, J. H.; Lee, K. J.; Kim, C. H.; Joo, T.; Hong, S.-C.; Cho, B. R. *J. Am. Chem. Soc.* **2008**, *130*, 4246-4247.
32. Turro, N. J., Ramamurthyl, V. & Scaiano, J. C. *Modern Molecular Photochemistry of*

Organic Molecules (University Science Books: Sausalito, California), **2010**, pp253-255

33. (a) Duhamel, J. *Langmuir* **2012**, *28*, 6527-6538. (b) Fujimoto, K.; Shimizu, H.; Inouye, M. *J. Org. Chem*. **2004**, *69*, 3271-3275. (c) Sau, S. P.; Hrdlicka, P. J. *J. Org. Chem*. **2012**, *77*, 5-16. (d) Ingale, S. A.; Pujari, S. S.; Sirivolu, V. R.; Ding, P.; Xiong, H.; Mei, H.; Seela, F. *J. Org. Chem*. **2012**, *77*, 188-199. (e) Wojciechowski, F.; Lietard, J.; Leumann, C. J. *Org. Lett*. **2012**, *14*, 5176-5179.
34. Hong, Y.; Lam, J. W. Y.; Tang, B. Z. *Chem. Soc. Rev.* **2011**, *40*, 5361-5388.
35. McRae, E. G.; Kasha, M. *L.G. Augenstein, B. Rosenberg, R. Mason (Eds.), Physical Processes in Radiation Biology, Academic Press, New York* **1964**, pp23-42.
36. Deniz, E.; Tomasulo, M.; Cusido, J.; Yildiz, I.; Petriella, M.; Bossi, M. L.; Sortino, S.; Raymo, F. M. *J. Phys. Chem. C* **2012**, *116*, 6058-6068.
37. Urano, Y.; Sakabe, M.; Kosaka, N.; Ogawa, M.; Mitsunaga, M.; Asanuma, D.; Kamiya, M.; Young, M. R.; Nagano, T.; Choyke, P. L.; Kobayashi, H. *Sci. Transl. Med.* **2011**, *3*, 110-119.
38. Pawlicki, M.; Collins, H. A.; Denning R. G.; Anderson, H. L. *Angew. Chem. Int. Ed.* **2009**, *48*, 3244-3266.
39. Williams-Harry, M; Bhaskar, A.; Ramakrishna, G.; Goodson, III, T.; Imamura, M.; Mawatari, A.; Nakao, K.; Enozawa, H.; Nishinaga, T.; Iyoda, M. *J. Am. Chem. Soc.* **2008**, *130*, 3252-3253.
40. Chung, S.-J.; Zheng, S.; Odani, T.; Beverina, L.; Fu, J.; Padilha, L. A.; Biesso, A.; Hales, J. M.; Zhan, X.; Schmidt, K.; Ye, A.; Zojer, E.; Barlow, S.; Hagan, D. J.; VanStryland, E. W.; Yi, Y.; Shuai, Z.; Pagani, G. A.; Brédas, J.-L.; Perry, J. W.; Marder, S. R. *J. Am. Chem. Soc*. **2006**, *128*, 14444-14445.
41. Tominaga, M.; Mochida, S.; Sugihara, H.; Satomi, K.; Moritomo, H.; Fuji, A.; Tomoyuki, A.; Suzuki, Y.; Kawamata, J. *Chem. Lett*. **2014**, *43*, 1490-1492.
42. Berezin, M. Y.; Zhan, C.; Lee, H.; Joo, C.; Akers, W. J.; Yazdanfar, S.; Achilefu, S. *J. Phys. Chem. B*. **2011**, *115*, 11530-11535.
43. Zhang, X.; Xiao, Y.; Qi, J.; Qu, J.; Kim, B.; Yue, X.; Belfield, K. D. *J. Org. Chem.* **2013**, *78*, 9153-9160.
44. Lee, S. K.; Yang, W. J.; Choi, J. J.; Kim, C. H.; Jeon, S-J.; Cho, B. R. *Org. Lett.* **2005**, *7*, 323-326.
45. Turro, N. J., Ramamurthyl, V. & Scaiano, J. C. *Modern Molecular Photochemistry of Organic Molecules* (University Science Books: Sausalito, California), **2010**, pp238, 277-287.

46. Turro, N. J., Ramamurthyl, V. & Scaiano, J. C. *Modern Molecular Photochemistry of Organic Molecules* (University Science Books: Sausalito, California), **2010**, pp303-304.
47. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy Third Edition*, Springer+Business Media, LLC; New York, **2006**, pp208-213.
48. Reichardt, C. *Chem. Rev.* **1994**, *94*, 231-2358.
49. Yamaguchi, Y.; Matsubara, Y.; Ochi, T.; Wakamiya, T.; Yoshida, Z. *J. Am. Chem. Soc.* **2008**, *130*, 13867-13869.
50. Figueira-Duarte, T. M.; Müllen, K. *Chem. Rev.* **2011**, *111*, 7260-7314.
51. (a) Mochida, K.; Kawasumi, K.; Segawa, Y.; Itami, K. *J. Am. Chem. Soc.* **2011**, *133*, 10716-10719. (b) Wu, J.; Pisula, W.; Müllen, K. *Chem. Rev.* **2007**, *107*, 718-747. (c) Yagi, A.; Venkataramana, G.; Segawa, Y.; Itami, K. *Chem. Commun.* **2014**, *50*, 957-959.
52. (a) Chen, R. J.; Zhang, Y.; Wang, D.; Dai, H. *J. Am. Chem. Soc.* **2001**, *123*, 3838-3839. (b) Nakashima, N.; Tomonari, Y.; Murakami, H. *Chem. Lett.* **2002**, *6*, 638-639.
53. Crawford, A. G.; Dwyer, A. D.; Liu, Z.; Steffen, A.; Beeby, A.; Pålsson, L. O.; Tozera, D. J.; Mardera, T. B. *J. Am. Chem. Soc.* **2011**, *133*, 13349-13362.
54. Zöphel, L.; Enkelmann, V.; Müllen, K. *Org. Lett.* **2013**, *15*, 804-807.
55. Saito, Y.; Shinohara, Y.; Ishioroshi, S.; Suzuki, A.; Tanaka, M.; Saito, I. *Tetrahedron Lett.* **2011**, *52*, 2359-2361.
56. Kim, H. M.; Lee, Y. O.; Lim, C. S.; Kim, J. S.; Cho, B. R. *J. Org. Chem.* **2008**, *73*, 5127-5130.

General Conclusion

1. Answer to the purpose in this thesis

As described in section 7.1. in General Introduction, the purpose in this thesis is to contribute to the development of 1) solvatochromic fluorophores with high brightness, photostability, and red-shifted absorption/fluorescence and 2) two-photon active fluorophores compatible with 1050 nm fiber lasers, by using pyrene as a key chromophore.

As for the former, the author has demonstrated that pyrene-based D-π-A fluorophores, *e.g.*, **PA** in chapter 2-2 and **PAD3** in chapter 3-1, can exhibit higher brightness than Prodan in both apolar and polar solvents. In addition, both **PA** and **PAD3** are compatible with visible light excitation unlike Prodan, exhibit stronger solvatochromism fluorescence than Nile Red, and show much higher photostability than Nile Red and Prodan. With respect to photostability, it is indicated that pyrene-based D-π-A fluorophores may possess the highest photostability among enormous solvatochromic fluorophores. These photophysical properties of **PA** and **PAD3** are derived from inherent nature of pyrene chromophore, especially its stable π–π* energy which has a key role in the suppression of intersystem crossing and subsequent enhancement of fluorescence quantum yield and photostability in apolar solvents.

Regarding the latter, the author has demonstrated that pyrene-based quadrupolar fluorophores such as pyrene-based A-π-A dye, **PY** in chapter 2-4, and pyrene-based A-π-D-π-A dye, **PYO2** in chapter 3-2, can exhibit strong fluorescence and large two-photon absorption within the biological window. Furthermore, both **PY** and **PYO2** possess great potential to be compatible with the 1050 nm fiber laser because their maximum wavelengths of two-photon absorption are around 1000 nm. These photophysical properties of **PY** and **PYO2** are also derived from inherent nature of pyrene chromophore such as centrosymmetric and planer structure, flexibility of molecular design, and large π electrons.

From these results, it can be concluded that the author has succeeded to accomplish the above-mentioned purpose in this thesis, that is, the development of the solvatochromic fluorophores and two-photon active dyes of which photophysical properties are not represented by existing molecules and urgently in demand from the view point of practical applications in biomedical field.

2. Scientific signification

Scientifically, the most important achievement in this thesis is to have connected the theory concerning photodegradation mechanism summarized by Branchard (see, section 5 in General Introduction) to the molecular design of solvatochromic fluorophores. Specifically,

the auther considered that the solvatochromic fluorophores showing inefficient intersystem crossing in apolar media would possess high photostability. Based on this assumption, the author succeeded to develop highly photostable solvatochromic fluorophores. So far, the photostability of the solvatochromic fluorophores have been not discussed well though it often becomes crucial drawbacks. Therefore, the consideration in this thesis would be useful to develop novel photostable solvatochromic fluorophores including not only ESICT but also ESIPT type fluorophores. In particular, the development of photostable ESIPT type molecule would be interesting because ESIPT type solvatochromic fluorophores possess much better ability to conduct quantitative analysis than ESICT type.

Next, it is also important to have demonstrated that pyrene is still attractive chromophore for the development of emitting materials, and therefore should be further investigated in the future. In this context, the established synthetic methodology of pyrene derivatives shown in this thesis also becomes valuable because the number of electronic donor and/or acceptor functionalized pyrene derivatives have been significantly limited. In fact, as shown in Figrure 15 in General Introduction, pyrene derivatives still have several structures to be studied. The author believes that further investigation of pyrene derivatives can overcome the remaining drawbacks of the pyrene dyes described in section 7.2. in General Introduction and subsequently contribute to the development of biology field.