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Fluorescence Detected Circular Dichroism for Highly Sensitive Observation of Exciton Coupled CD

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Summary. The fluorescence detected circular dichroism (FDCD) appearance of some exciton coupling compounds is reviewed by a newly developed ellipsoidal mirror system (FDCD465). The FDCD465 achieves both the complete elimination of the polarization artifact and a dramatic enhancement of detection sensitivity expanding the applicability of exciton coupling fluorophores. A variety of chromophores that possess large extinction coefficients and high fluorescence quantum yields are available for exciton coupled FDCD, regardless of the degree of fluorescence polarization. Different types of FDCD devices can regulate FDCD polarization artifacts and the photoselection effect, enabling effective surveys of FDCD potential.

Keywords. Chirality; Circular dichroism; Configuration; Chromophores; Spectroscopy.

Introduction

Circular dichroism (CD) [1], the difference in the absorption of left- and right-circularly polarized light, is an important analytical method frequently used to elucidate the absolute configurations of small molecules [2, 3] or to probe the conformation and interactions of biochemical systems [4]. The analyses are generally simple and quick to perform and the analytical strategies are corroborated by both theory and empirical rules.

For fluorescent samples, the difference in CD also can be measured by detecting fluorescence intensity. This differential fluorescence measurement is called fluorescence detected circular dichroism (FDCD) [5] and possesses several advantages over transmission CD [6]. The specificity of fluorescence enhances the usefulness of conformation-sensitive CD, enabling CD measurement of only the fluorescent portions of a solution mixture or macromolecule [5]. In general, fluorescent

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measurements can be performed at much lower concentrations than transmission CD measurements [7]. In principle, FDCD can determine the CD along a specific transition moment in certain molecules in rigid systems [8] and can obtain stereochemical information even from highly scattering or opaque solutions [9].

This report provides an introduction and recent observations of the FDCD method, including the development of FDCD, instrumental considerations, and application to exciton chirality. The experimental section presents a practical guide for FDCD measurements using the newest ellipsoidal mirror attachment JASCO FDCD465 instrument [10].

FDCD Development

The first report of FDCD [5] has provided a simple schematic and an extraction of the CD curve from a solution mixture. The CD curve of the fluorescent amino acid *L*-tryptophan has been measured selectively from a mixture with *L*-cystine, indicating that the method is a powerful technique for investigating local structures within a macromolecule. The definition of FDCD has been expanded theoretically to show its relation to transmission CD and applied to the two-state conformational analysis of flexible molecules in solution [11]. Reports [8, 12] also note that interpretation of FDCD results requires care when rotatory *Brownian* motion is frozen or restricted within the fluorescence lifetime of the chromophore used, because the electric dipole transition moments of the absorption and emission bands may not be parallel or perpendicular to each other. This is defined as the photoselected case and is distinguished from the isotropic case for which the measured fluorescence intensity of the chromophore depends exclusively on the amount of light absorbed. Theoretical treatment of FDCD in photoselected cases has been described successfully as a direction-dependent optical activity [8]. From a practical viewpoint, however, polarization of emitted light is not fully distributed to the photoselection, and unwanted artifacts occur in FDCD experiments of polarized cases. These FDCD artifacts have been eliminated by placing a linear polarizer (at a 90° angle) between the cell and photomultiplier tube (PMT) [13] to yield the photoselected FDCD of *d*-10-camphorsulfonic acid in glycerol, or by utilizing a two-PMT system to afford the FDCD of morphine in 90% glycerol [14] and of several proteins [15]. More advanced FDCD techniques have been developed based on basic principles. For example, multi-dimensional FDCD [16, 17] enables extremely selective and sensitive measurements, and lifetime-resolved FDCD [18, 19] identifies the components of a solution or molecule accurately. However, these advanced FDCD methods have not been widely implemented.

Instrumental Considerations for FDCD

Four distinct setups have been reported for measuring FDCD, which apply to the sample chamber of a standard CD spectrometer, except for the two-PMT system that requires a modified Cary 60 spectrometer to obtain artifact-free FDCD [15]. The primary setup adopted in the first FDCD report [5] was 90° collection with a one-PMT system. Emission is detected at 90° to the direction of excitation light through a long-pass filter in front of the detector (Fig. 1a). This setup is provided

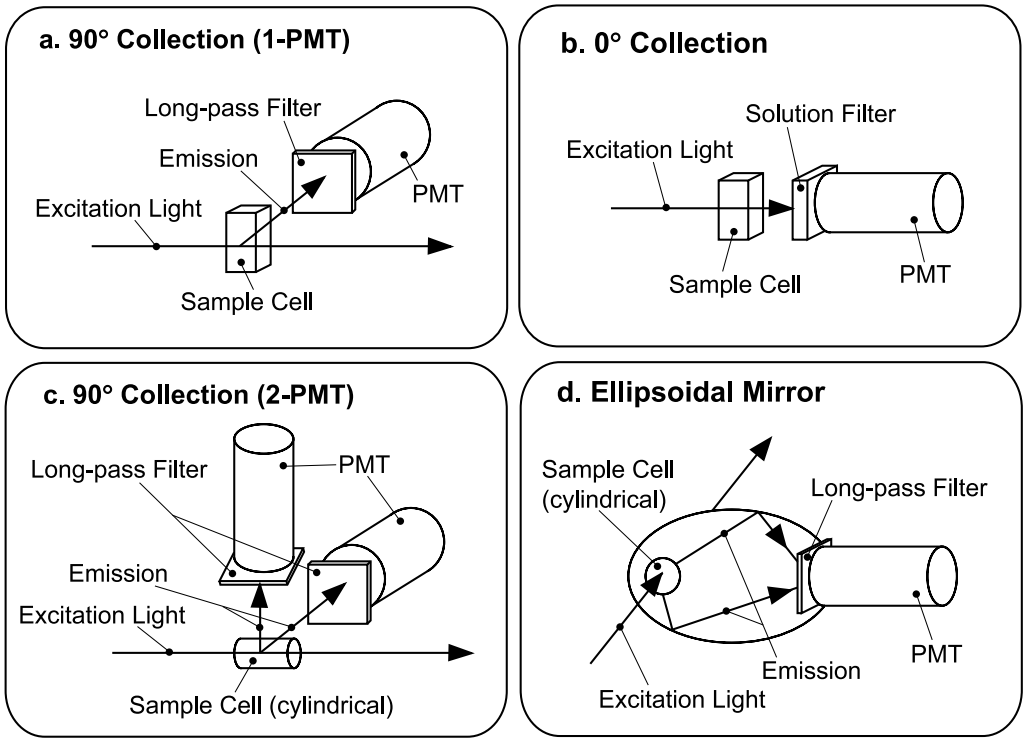

Fig. 1. Schematic representation of four distinct FDCD setups; excitation light is left- and right-circularly polarized; (a) 90° collection with one-PMT system; (b) 0° collection; (c) 90° collection with two-PMT system; (d) ellipsoidal mirror (all directions)

by the JASCO FDCD405, which contains a standard fluorescence square cell and works well for isotropic samples [7], but FDCD of polarized samples may be affected by artificial signals caused by coupling between the fluorescence polarization and polarized light imperfections [20]. This artifact was avoided by a simple setup, resulting in photoselected FDCD [12]. As shown in Fig. 1b, a PMT at 0° to the incident light detects emissions with help from a solution filter that eliminates undesired transmission of excitation light. Although this artifact-free measurement has been utilized in some situations [21, 22], its use is limited by the difficulty choosing an optimal solution filter. The FDCD polarization artifact can be avoided if a linear polarizer at the appropriate orientation is employed in front of the PMT at 90° [13]. This facile solution allowed successful measurement of photoselected FDCD [13], but the linear polarizer diminished the detected light so that detection sensitivity was significantly reduced [23]. Protein FDCD [15] did not involve artifacts when the light was collected by two PMTs at 90° as depicted in Fig. 1c. This method, however, has not been further developed because an appropriate instrumental environment is difficult to create. Consequently, the system that satisfies the principles of ideal light collection is an ellipsoidal mirror arrangement [24], which enables artifact elimination and sensitivity enhancement by collecting nearly all the emitted light. Although the original ellipsoidal mirror system appeared intricate, a practical alternative, the JASCO FDCD465, has been

reported recently [10]. This simple ellipsoidal mirror attachment is completely compatible with a standard CD spectrometer and is expected to promote the potential of FDCD in many applications.

Application of FDCD to the Exciton Chirality Method

Through instrumental development, FDCD has been expected to function mainly as an analytical probe for macromolecules [25] because the principal contributors to FDCD development were those involved in macromolecular research. However, progress has been also made in transmission CD development. The more reliable photo-elastic modulator (PEM) [26] has replaced the *Pockels* cell as a generator of elliptically polarized light. Computer-assisted operation and data processing have been incorporated into CD systems. An easy and reliable calibration method has been established [27], allowing quantitative analyses of CD data. Especially, the CD exciton chirality method has been recognized as a useful application of CD [1, 28].

The CD exciton chirality method, based on simple dibenzoate rules and quantum mechanical coupled oscillator theory, is probably one of the methods most fruitful in FDCD. In fact, FDCD was successfully applied to compounds possessing two or more identical fluorophores that have absorptions strong and sharp enough to allow exciton coupling, demonstrating the existence of favorable conditions for agreement between FDCD and transmission CD with a sensitivity enhancement of 50–100 fold [7]. The potential of this exciton-coupled FDCD has been further investigated with exciton-coupling systems containing a variety of chromophores used in transmission CD studies. One study has revealed that optimal fluorophores for the method possess a large extinction coefficient, high fluorescence quantum yield, and negligible polarization of fluorescence (P_F). When the scope of exciton-coupled FDCD was surveyed in the study [20] using a 90° collection with one-PMT system, two distinct cases were revealed based on the extent of agreement between the spectra obtained from FDCD and from transmission CD. Good agreement was observed between FDCD and CD when the degree of P_F was negligible, and the exciton-coupled FDCD spectrum deviated significantly from transmission CD when the degree of P_F was relatively large. The new ellipsoidal FDCD465 has established a new record for detection sensitivity by 20 times compared to the previous FDCD405 [10], even if fluorescence polarization is purposely induced. FDCD for a polarized solution of a simple exciton coupling compound in glycerol/*Me*OH was measured successfully at concentrations as low as $10^{-9}\,M$ [10]. This report investigates factors of exciton-coupled FDCD that conduce application of the method and further knowledge of the new ellipsoidal FDCD465.

Results and Discussions

To investigate the properties of accessible FDCD devices, the ability of FDCD to control photoselection effects and polarization artifacts is reviewed for the JASCO FDCD405 (Fig. 1a, 90° collection with one-PMT) with and without a polarizer [23] and FDCD465 (Fig. 1d, ellipsoidal mirror) [10]. As listed in Table 1, FDCD405 eliminates polarization artifacts when used with an 81–90° polarizer [23].

Table 1. Features of accessible FDCD devices

Device	Polarization artifact	Photoselected FDCD	Detection sensitivity
FDCD405 (no polarizer)	out of control	out of control	high[a]
FDCD405 (polarizer at 81–90°)[b]	eliminate	out of control[c]	low[a]
FDCD465 (ellipsoidal mirror)	eliminate	eliminate	high[a]

[a] in comparison with conventional CD; [b] the angle of polarizer is experimentally adjusted to diminish the fluorescence polarization; [c] at the polarizer angle 81–90°, where the polarization artifact is eliminated, the obtained FDCD is photoselected

The photoselection effect theoretically can be regulated by FDCD405 with a polarizer [8], where certain angle (35.25°, the magic angle) diminishes the effect, but allows observation of a photoselected FDCD of only a particular angle (90°) that produces no polarization artifact [13]. This anisotropic issue is observed when both transition vectors between absorption and emission are not parallel [8], but detection sensitivity is inevitably sacrificed when a polarizer is added. This sensitivity drop may be significant when fluorescence quantum yield is not high, as for a compound with modest extinction coefficients. The ellipsoidal FDCD465 always affords isotropic FDCD that in principle eliminates the photoselection effect and polarization artifacts. Isotropic FDCD is different from photoselected FDCD if the photoselection effect is significant, as demonstrated in a preliminary experiment using (*R*)- and (*S*)-1,1′-bis-2-naphthol [29].

The features of available FDCD devices were confirmed by ethylene glycol solutions of (1*R*,2*R*)- and (1*S*,2*S*)-*trans*-1,2-cyclohexanediol 1,2-bis(4-phenylbenzoate) (**1**). In this case, the viscosity of the solvent amplifies solution anisotropy, leading to large polarization artifacts [20]. Figure 2A and B demonstrate changes in the degree of P_F and FDCD polarization artifacts, when a linear polarizer was introduced on the FDCD405. The extracted polarization of emission was maximized when the angle of polarizer was 0° (solid line) and minimized when the angle was 90° (gray dotted line). The angle that minimizes artifact formation is determined experimentally by monitoring the fluorescence polarization. Measurement with no polarizer (dotted line) is considered the average of all angles. The change in degrees of P_F (Fig. 2A) corresponds well to the polarization artifact in FDCD raw data (Fig. 2B). The FDCD curve obtained by either FDCD405 with a 90° polarizer (not shown) or FDCD465 (ellipsoidal, gray lines) resulted in FDCD curves with an identical shape as transmission CD (solid lines) with a satisfactory S/N ratio, as shown with $\Delta\varepsilon$ in Fig. 2C and D. The ellipsoidal FDCD465 successfully eliminated polarization artifacts with reasonable sensitivity, yielding isotropic FDCD. This result coincides with reports involving the ellipsoidal FDCD465 [10], where both artifact elimination and sensitivity enhancement have been achieved.

To determine the scope of the exciton-coupled FDCD method, a library of exciton coupling compounds has been explored using a prototype of FDCD405

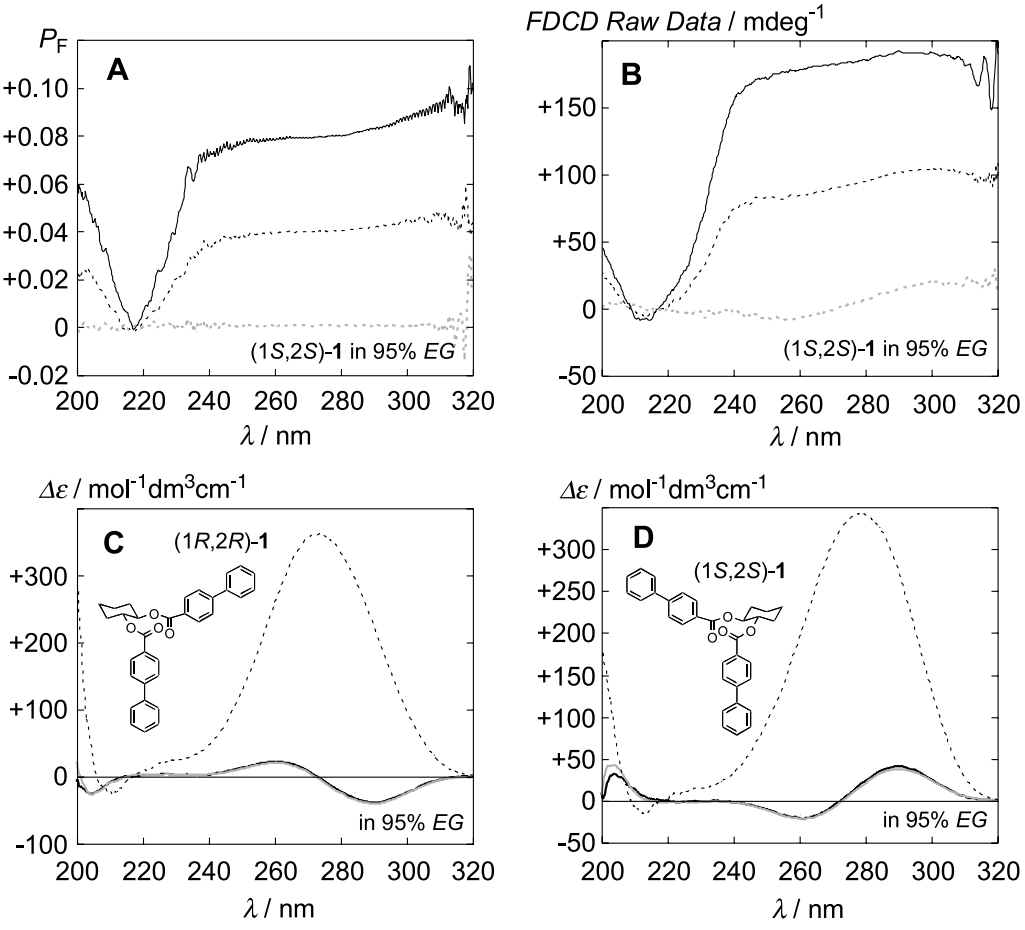

Fig. 2. Fluorescence polarization spectra (**A**), FDCD raw data (**B**), and FDCD/CD curves in $\Delta\varepsilon$ (**C** and **D**) of *trans*-1,2-cyclohexanediol 1,2-bis(4-phenylbenzoate) (**1**) in 95% ethylene glycol (*EG*); (1*S*,2*S*)-**1** in 4.56 μM was used for **A**, **B**, and **D**; (1*R*,2*R*)-**1** in 4.58 μM was used for **C**; curves shown in **A** and **B** were obtained by FDCD405 with no polarizer (artifact not eliminated, dotted lines), with 0° polarizer (artifact maximized, solid lines in **A** and **B**), and 90° polarizer (artifact eliminated, gray dotted lines); curves in **C** and **D** were FDCD using FDCD405 with no polarizer (dotted lines), using FDCD465 (ellipsoidal mirror, gray solid lines), and transmission CD (solid lines)

with no polarizer [20]. In this report, FDCD applications are reviewed with FDCD devices, both the traditional FDCD405 with and without a polarizer and the new ellipsoidal FDCD465, employing acetonitrile solutions of model compounds in both enantiomeric forms. All the compounds tested were selected from a previously reported compound lineup [20], revealing that the cases involving FDCD/CD deviations can be assigned into three groups as follows.

The first group is represented by compound **1**, which possesses two identical fluorophores. The fluorescence properties of 4-phenylbenzoate, including the reported fluorescence lifetime of 0.8 ns and estimated rotational correlation time of 0.031 ns for the single chromophore [20], indicate that compound **1** accompanies the FDCD polarization artifact (see Fig. 3) when measured by FDCD405 with no polarizer (dotted lines), *i.e.*, this is a case involving polarization in which the

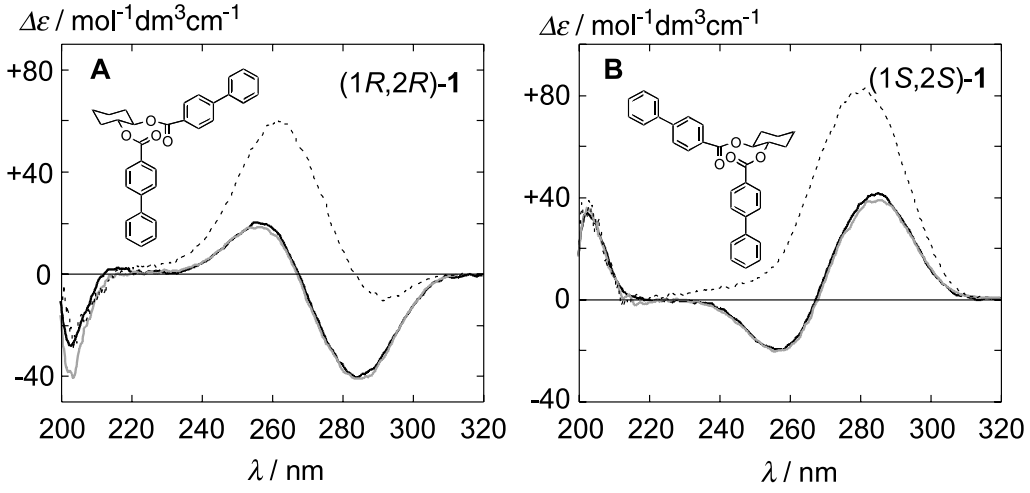

Fig. 3. CD/FDCD curves of *trans*-1,2-cyclohexanediol 1,2-(4-phenylbenzoate) (**1**) in acetonitrile; (**A**) (1*R*,2*R*)-**1** in 4.58 μM; (**B**) (1*S*,2*S*)-**1** in 4.81 μM; CD (bold solid lines), FDCD obtained by FDCD405 with no polarizer (dotted lines), and FDCD by ellipsoidal FDCD465 (gray solid lines)

excited chromophore emits before the compound is completely randomized [12]. FDCD measurement of both enantiomers by FDCD405 with no polarizer (dotted lines), which should yield mirror images, also supports the existence of artifacts. For each enantiomer, the FDCD curve agreed completely with transmission CD (solid line) when either a linear polarizer (90°, not shown) on FDCD405 or an ellipsoidal FDCD465 (gray line) was employed. This result indicates that the ellipsoidal FDCD465 can expand the applicability of fluorophores in exciton-coupled FDCD, since it always excludes both the polarization artifact and photoselection effect, regardless of fluorophore polarization.

The appearance of a second group is demonstrated using *trans*-1,2-cyclohexanediol 1-(4-bromobenzoate)-2-(2-naphthoate) (**2**), which contains fluorescent 2-naphthoate and nonfluorescent 4-bromobenzoate. Fluorescent 2-naphthoate is considered an isotropic fluorophore that yields a negligible degree of P_F (0.0032) for a single chromophoric form [20]. As depicted in Fig. 4, the FDCD curve of **2** obtained by FDCD405 with no polarizer (not shown) is identical to that produced by FDCD465 (gray lines), which always affords isotropic spectra. Thus, these results demonstrate that both FDCD spectra are isotropic (not photoselected) and free of polarization artifacts. However, the red shift of extrema and the decrease in intensity of both FDCD spectra, while maintaining the overall bisignate shape, are apparent in comparison to transmission CD (solid lines). The cause of the deviation in spectral shapes between FDCD and transmission CD is unclear, but may be attributed to energy transfer [30] and/or a new phenomenon, because these FDCD spectra are artifact-free and not photoselected. As the overall bisignate shape of the FDCD curve was maintained, this case may provide accurate information about stereochemistry.

The third group is described by *trans*-1,2-cyclohexanediol 1-(4-bromobenzoate)-2-(4-phenylbenzoate) (**3**), which consists of the polarized fluorophore 4-phenylbenzoate and the nonfluorophore 4-bromobenzoate, and illustrates poor

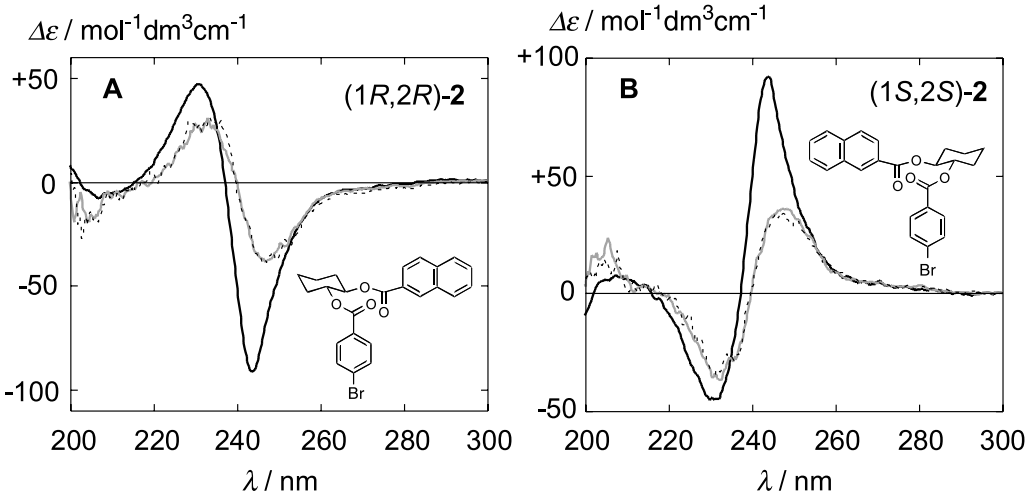

Fig. 4. CD/FDCD curves of *trans*-1,2-cyclohexanediol 1-(4-bromobenzoate)-2-(2-naphthoate) (**2**) in acetonitrile; (**A**) (1*R*,2*R*)-**2** in 2.90 μM; (**B**) (1*S*,2*S*)-**2** in 2.95 μM; CD (bold solid lines), FDCD obtained by FDCD405 with no polarizer (dotted lines), and FDCD by ellipsoidal FDCD465 (gray solid lines)

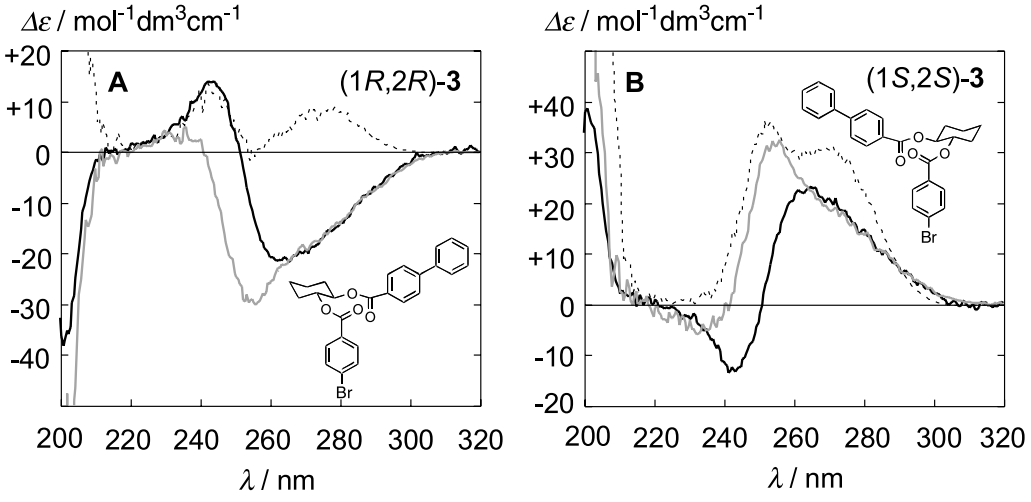

Fig. 5. CD/FDCD curves of *trans*-1,2-cyclohexanediol 1-(4-bromobenzoate)-2-(4-phenylbenzoate) (**3**) in acetonitrile; (**A**) (1*R*,2*R*)-**3** in 8.22 μM; (**B**) (1*S*,2*S*)-**3** in 7.99 μM. CD (bold solid lines), FDCD obtained by FDCD405 with no polarizer (dotted lines), and FDCD by ellipsoidal FDCD465 (gray solid lines)

agreement between FDCD and CD [20]. Actually, the polarization artifact caused deformation of the FDCD curves for both enantiomers as depicted by dotted lines in Fig. 5 (FDCD405 with no polarizer). These FDCD polarization artifacts were successfully mitigated by FDCD465 (gray lines), but the resultant FDCD curves still deviate from conventional CD (solid lines). The cause of this deviation is unclear, but also may be an effect such as energy transfer and/or a new phenomenon, as discussed for **2**. If a natural product of interest possesses a combination of

chromophores equivalent to those in this case, the position of the FDCD peaks may be assigned carefully and an accurate stereochemical analysis obtained, otherwise 4-phenylbenzoate may not be recommended for objective derivatizations, nor is nonfluorescent *p*-bromobenzoate. This example shows that the newly developed FDCD465 has increased the application of FDCD experiments.

In conclusion, FDCD can provide accurate chiral assignments when combined with CD exciton chirality methods, especially in lower concentrations. The ellipsoidal FDCD465, a universal device that works on standard CD instruments, functions to exclude the FDCD polarization artifact with enhanced detection sensitivity by collecting almost all the emitted light. The detection sensitivity of FDCD465 can be 3.5 times higher than the previous FDCD405 device and more than 100 times greater than transmission CD, allowing measurements at as low concentration as $10^{-9}\,M$ in favorable cases [10]. The controllability of FDCD polarization artifacts and photoselection effects using different types of FDCD devices (FDCD405 with/without a polarizer) in addition to the ellipsoidal FDCD465 enables studies that extend the application of FDCD.

Experimental

All compounds for FDCD measurements were prepared as described previously [20]. The solvents used in FDCD measurements include acetonitrile (OmniSolv, spectrophotometric grade) and ethylene glycol (Wako Pure Chemical, S grade). UV/Vis spectra were recorded on a JASCO V-560 instrument. CD/FDCD measurements were performed on a JASCO J-820 spectropolarimeter. To ensure that any observed difference between FDCD and CD was not derived from sample preparation, identical solutions were used for both FDCD and CD measurements in all cases. Re-absorption of emitted light was avoided by adjusting the absorbance of all solutions for measurements to 0.2 or lower. This adjustment was based on a previous report [20] demonstrating that compounds with large *Stoke*'s shifts exclude strong overlaps between excitation and emission.

Fluorescence Properties

Fluorescence excitation and emission were recorded on a JASCO FP-6500 instrument. Fluorescence polarization spectra, an indicator of FDCD polarization artifact, were recorded on a JASCO J-820 spectropolarimeter attached to an FDCD405 device that was previously calibrated with *Norden*'s LD-calibrator [10, 31]. The signals obtained in LD-mode were converted to the degree of polarization by multiplying by $\ln 10/2 = 1.1513$, affording a reasonable degree of fluorescence polarization [10].

FDCD Measurements

Two types of FDCD devices were included in this work. The FDCD405 device has been developed in 2001 as a high-sensitive attachment for the JASCO J-800 type CD spectropolarimeter [23], which employs a removable linear polarizer at an angle of 81–90° for regulating polarization in the detected fluorescence. The device features a square fluorescence cell and collects emitted light at 90° with respect to the excitation. Measurements by FDCD405 with no polarizer may produce an FDCD curve with a polarization artifact when the solution is polarized during fluorescence. FDCD465 is a newly developed ellipsoidal mirror device [10], which allows isotropic FDCD, excluding both the polarization artifact and photoselection effect. The appropriate orientation of the polarizer on FDCD405 and the optimal pair of balancing masks on FDCD465, which yield the lowest degree of fluorescence polarization, were obtained by monitoring P_F with the linear dichroism mode of the J-800 series

[10, 32, 33]. In all FDCD measurements, transmitted light was eliminated by placement of a *Rayleigh* horn beam trap, while scattered light was prevented by employing a long-pass filter in front of the PMT. The choices of long-pass filters for FDCD measurements, which control the cutting edges of the detected emissions, were made based on the fluorescence excitation and emission spectra. The appropriate filter prevented scattering of the excitation light and maximized the emitted light signal. The FDCD spectrum was calibrated with an ammonium d-10-camphorsulfonate aqueous solution [27] placed in the path of the excitation beam before the fluorescence cell filled with an achiral fluorescent dye [15].

Recording the difference in emission intensity over excitation by left- and right-circularly polarized light yielded an FDCD spectrum as described by $S = k(F_L - F_R)/(F_L + F_R)$, where F_L and F_R are expressed in relative intensities and $(F_L + F_R)$ was measured as DC voltage in volts. The derived instrumental constant $k(+28648)$ refers to milli-ellipticity [10], which is measured in millidegrees (mdeg). The FDCD raw data (S) were converted into corresponding CD spectra according to the equation: $\Delta\varepsilon = \varepsilon_L - \varepsilon_R = ((3.032 \times 10^{-5})S(1 - 10^{-A}))/(cd10^{-A})$, where A is UV absorbance, c is molar concentration (M), and d is cell length (cm). This conversion process, including the sign and value of the instrumental constant, is standard when applying FDCD to the exciton chirality method, and allows direct comparison between shapes of the FDCD spectra and transmission CD [7, 30].

For **3**, the efficiency of artifact elimination varied by wavelength; no pair of balancing masks completely eliminated the polarization. The residual FDCD polarization artifact was eliminated completely by utilizing the artifact-estimation-factor (F_{art}) [10], which converts fluorescence polarization (P_F) into FDCD polarization artifact using an empirical equation (artifact $= P_F \times F_{art}$). Since this factor is an inherent value of the CD spectrometer, it needs to be independently determined from an ethylene glycol solution of sodium fluorescein, a standard solution of an achiral molecule under polarized conditions. This method enables artifact estimation without needing a racemic solution of the enantiomers. The true FDCD of optically active **3** was then obtained by subtracting the estimated FDCD artifact from the apparent FDCD data.

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