Polarization resolved microscopy techniques, recently introduced in nonlinear optical processes such as two photon excited fluorescence [1], second-order harmonic generation [2,3], and more recently, coherent anti-Stokes Raman scattering [4], have shown their potential to provide extensive structural information in molecular and biomolecular samples, with the possibility of differentiating between single crystals and noncrystalline materials [3]. Implementing such optical diagnostics for detecting protein crystals is useful for screening crystallization conditions on small-size crystals prior to structural studies by x-ray crystallography [5]. Detecting proteins without staining, however, requires addressing fluorescence from tryptophan or other amino acids in UV excitation or detection range conditions, both of which are poorly adapted to imaging. A configuration using two photon excitation has recently been implemented, which requires high-power, visible range-pulsed laser sources [6].

In this Letter, we show that polarization resolved three photon excitation fluorescence (3PEF) microscopy can be successfully implemented with standard Ti:sapphire femtosecond laser excitation and used as a diagnostic for crystallinity in protein samples. A three photon excitation of the UV transition of amino acids is generated by a near-IR illumination in a conventional multiphoton microscope [7,8]. Furthermore, polarization resolution brings two additional features. First, its sensitive angular photoselection allows for the detection of structures exhibiting up to sixfold symmetry [9], which is appropriate for crystals. Second, depolarization produced by Förster resonance energy transfer between nearby molecules (homo-FRET) [10] in a crystal can be readily detected and used as a diagnostic for the presence of defects at the nanometric scale of different orientation or symmetry. Homo-FRET has been successfully used to reveal local interaction between proteins in nanoscale clusters [11,12], a property that we exploit here in crystals. We first validate this technique on a well-known 1,4-diphenylbenzene (p-terphenyl) molecular crystal, active in the same excitation and emission wavelength range as tryptophan. We then investigate polarization resolved 3PEF responses from protein single crystals, using a well-studied model system (lysozyme).

The 3PEF polarization resolved analysis is based on a Ti:sapphire laser beam excitation (150 fs pulse duration at a repetition rate of 80 MHz) set at an 840 nm incident wavelength and focused on the sample by a high numerical aperture objective (× 60, 1.2 NA). The continuous rotation of the linear incident polarization in the sample plane gives access to polarimetric information. The fluorescence signal in the range of 280–380 nm is collected in the forward direction by a UV objective (× 40, 0.6 NA), filtered by dichroic mirrors and bandpass filters, and directed to a UV polarization beam splitter that separates the beam toward two photomultipliers sensitive in the UV range. Thus, the fluorescence emission is detected simultaneously in two orthogonal polarization directions with intensities denoted \( I_X \) and \( I_Y \).

Polarization resolved fluorescence responses in crystals are governed by their symmetry, the presence of homofRET between closely spaced molecules, and the possible presence of defects. First, crystal symmetry has a strong effect on the shape of polarization responses in fluorescence. Typical theoretical polarization responses in one photon fluorescence (1PEF) and 3PEF are shown in Fig. 1 as polar graphs for a crystalline structure made of up to four transition dipole directions \( \mathbf{\mu}_{n=1-4} \) lying in the \((X, Y)\) sample plane. As in previous work along the same lines on two photon fluorescence [1,3], the polarization responses are built by incoherent addition of the fluorescence intensities from each dipole orientation, accounting for absorption and emission probabilities (here, absorption
measurements in solution have shown that strong homo-
In proteins, including lysozyme, fluorescence anisotropy
is, proportional to $I_{22}^{E}(r) = C_24 \nu^2$ one can thus expect close to
molecules [13,14]. In single crystals where the distance between
(responses (f)) The 3PEF responses for a pure fourfold symmetry and (g) pure sixfold symmetry. (h) The
3PEF responses of pure fourfold (or sixfold) symmetry structures
for the 1PEF contrast with a polarization insensitive
response, with a more or less pronounced symmetry depend-
least four dipoles lead to a four-lobe pattern 3PEF re-
3PEF signal [9]. The comparison of 1PEF and 3PEF shows
for the 1PEF contrast with a polarization insensitive
shape of 3PEF is sensitive to the symmetry of the structure up to the 6th
order, any symmetry order above two is seen as isotropic
for the 1PEF contrast with a polarization insensitive ($I_x + I_y$) response [Fig. 1(b)]. The 3PEF polarization re-
sponds of pure fourfold (or sixfold) symmetry structures are characteristic with four-lobe (or six-lobe) shapes
[Figs. 1(f) and 1(g)]. Any other symmetries formed by at least four dipoles lead to a four-lobe pattern 3PEF re-
sponse, with a more or less pronounced symmetry depending on the 3D orientation of the crystal (data not shown).
The 3PEF polarization responses are therefore indicative of the minimum number of dipoles in the crystal unit cell, which contribute to the fluorescence response. Second, homo-FRET (and possibly other short-range, strong coupling energy-transfer mechanisms) can lead to a depolarization of the emitted fluorescence. Homo-FRET is known to be significant in molecular crystals due to the very close distance between dipoles, with typical 50% efficiency reached at a distance of $R_0 \sim 40$–100 Å between mole-
cules [13,14]. In single crystals where the distance between molecules is very small ($\sim 5$ Å in $p$-terphenyl crystals), one can thus expect close to 100% homo-FRET efficiency. In proteins, including lysozyme, fluorescence anisotropy measurements in solution have shown that strong homo-
FRET between intraprotein tryptophan residues occurs already at distances up to 12 Å [15–18]. Interprotein
homo-FRET between tryptophan and/or other amino-acid residues has also been evidenced, with significant efficiencies in protein aggregates [11,12]. In order to account for this radiationless transfer of energy from an excited donor to an acceptor, the orientation-dependent coupling term $\kappa^2$ stemming from the dipole-dipole interaction potential be-
tween nearby molecules [10] has to be introduced in the fluorescence intensity. Denoting $T$, the transfer efficiency factor, which depends essentially on the distance between neighbor molecules and their absorption-emission spectral overlap, the total fluorescence intensity analyzed along the $J$ direction can be written
$I_J^{PE}(\alpha) = (1 - T) I_J(\alpha) + T I_J^{FRET}(\alpha)$, with $I_J^{FRET}(\alpha)$ the homo-FRET contribution:

$$I_J^{FRET}(\alpha) \propto \sum_{\Omega_{a=1-N}} \sum_{\Omega_{b=1-N}} \sum_{\Omega_{ab}} \left| \mathbf{\mu}(\Omega_{a}) \cdot \mathbf{E}(\alpha) \right|^6$$

$$\times \left| \mathbf{\mu}(\Omega_{b}) \cdot \mathbf{J} \right|^2 \kappa^2(\Omega_{ab}, \Omega_{a}, \Omega_{b}),$$

(1)

with $\mathbf{\mu}(\Omega_{a}) = \mathbf{\mu}_{a}$ and $\mathbf{\mu}(\Omega_{b}) = \mathbf{\mu}_{b}$ the donor and accep-
tor dipoles of orientations $\Omega_{a} = (\theta_{a}, \phi_{a})$ and $\Omega_{b} = (\theta_{b}, \phi_{b})$ in the macroscopic frame, and $N$ the number of excited dipoles in the focal volume. In the macroscopic frame, $\Omega_{ab} = (\theta_{ab}, \phi_{ab})$ is the orientation angle of the vector $\mathbf{r}_{ab}$ between the donor and acceptor dipoles. The energy transfer near-field dipole-dipole coupling coeffi-
cient, normalized to 1 in Eq. (1), is $\kappa^2(\Omega_{ab}, \Omega_{a}, \Omega_{b}) \propto \left| \mathbf{\mu}_{a} \cdot \mathbf{\mu}_{b} - 3(\mathbf{\mu}_{a} \cdot \mathbf{r}_{ab})(\mathbf{\mu}_{b} \cdot \mathbf{r}_{ab}) \right|^2$.

In a crystal where neighboring molecules are closely packed, the $\mathbf{r}_{ab}$ vector directions are likely to span all the 3D space in a first approximation, leading to a considerable simplification of the above expression. Indeed, after averaging over $\Omega_{ab}$, the homo-FRET con-
tribution can be written as the product of two functions of separable variables $\Omega_{a}$ and $\Omega_{b}$, a consequence of the
complete decorrelation between the excitation and emission processes:

\[ I_{\text{RET}}^f(\alpha) \propto f(\alpha)g_J, \tag{2} \]

where the proportionality coefficient contains the average of \( \kappa^2 \) over \( \Omega_{ab} \), \( f(\alpha) = \sum_\Omega |\mu(\Omega_a)E(\Omega)|^2 \) and \( g_J = \sum_\Omega |\mu(\Omega_b)J|^2 \). Equation (2) leads to \( I_{\text{RET}}^f(\alpha) \propto \frac{I_{\text{RET}}^f(\alpha) - I_{\text{RET}}^f(\alpha)}{I_{\text{RET}}^f(\alpha) + I_{\text{RET}}^f(\alpha)} \); therefore, in the case of a complete transfer process, the 3PEF polarization response only is sensitive to the excitation photoselection \( f(\alpha) \), its amplitude being governed by the emission probability \( g_J \). Thus, for \( T = 1 \), we expect identical polar-graph shapes for the polarization responses \( I_{\text{RET}}^f(\alpha) \) and \( I_{\text{RET}}^f(\alpha) \), as illustrated in Figs. 1(d) and 1(e). To quantify the departure from the property \( I_{\text{RET}}^f(\alpha) \propto I_{\text{RET}}^f(\alpha) \), an anisotropy factor \( A(\alpha) = (I_{\text{RET}}^f(\alpha) - I_{\text{RET}}^f(\alpha))/(I_{\text{RET}}^f(\alpha) + I_{\text{RET}}^f(\alpha)) \) is introduced, which is expected to be a constant quantity with respect to \( \alpha \) when complete transfer (\( T = 1 \)) occurs. Any departure from this situation leads to a variation quantified by its standard deviation \( \sigma_A \). Figure 1(h) shows that for the case of Fig. 1(a), \( \sigma_A \) becomes negligible compared to typical experimental noise (\( \sigma_A < 0.05 \)) for \( T > 0.70 \), which is expected to be reached in crystals \([11-18]\). Assuming that the parameter \( r_{ab} \) explores all directions of the 3D space is not rigorously correct in crystals; the positions of nearby neighbors are in well-defined discrete positions. Accounting for neighbors in various positions, Eq. (1) shows that when more than four acceptor dipoles are present around a donor dipole, the proportionality property indicated is not strongly modified, no matter what the initial crystal symmetry [Fig. 1(h) illustrates the case of eight neighbors]. Finally, the hypothesis of parallel absorption and emission dipoles might be erroneous for some molecules: simulations show, however, that while this angle can considerably modify the \( I_{\text{RET}}^f(\alpha) \) and \( I_{\text{RET}}^f(\alpha) \) responses, the depolarization effect depicted in Fig. 1(h) does not significantly change, whatever the value of this angle.

The proportionality property \( I_{\text{RET}}^f(\alpha) \propto I_{\text{RET}}^f(\alpha) \) is no more valid in crystals exhibiting structural defects. If the scale of these defects is larger than \( R_0 \), but still smaller than the focal volume of the microscope objective (typically 300 nm lateral size), then the resulting intensity is transformed into the incoherent sum of the contributions of different defect regions of different crystalline orientations or symmetries, which do not undergo homofRET coupling: \( I_{\text{RET}}^f(\alpha) \propto I_{\text{RET}}^f(\alpha) + \cdots I_{\text{RET}}^f(\alpha) \), with \( I_j \) the fluorescence-polarization response of the defect region number \( i = 1, \ldots, n \). In this case, the polarization responses \( I_{\text{RET}}^f(\alpha) \) and \( I_{\text{RET}}^f(\alpha) \) no longer have similar shapes, as usually observed in diluted disordered media \([1]\). This property can be used as a diagnostic for crystallinity, provided that \( T > 0.70 \), for structural defects of size between 1 and 100 nm.

As a model crystal, we have selected \( p \)-terphenyl, with a structure that has been widely studied by x-ray diffraction \([19]\). This crystal is monoclinic (\( P2_1/a \) space group, corresponding to the \( C_2 \) point group for optical analysis) at room temperature with two molecules per unit cell \([20]\). The long molecular axis of \( p \)-terphenyl lies along the crystal axis 3 [Fig. 2(a)]. The organization of molecules in the unit cell, with central rings tilted with respect to the outer rings [Fig. 2(b)], leads to four possible transition dipole-moment directions \( \mu \) in the (1, 2) plane and an additional one along the 3 direction [Figs. 2(a) and 2(b)]. The orientation of this unit cell in the macroscopic frame is defined by the Euler set of angles \( (\theta, \phi, \psi) \) [Fig. 2(c)]. All 3PEF polarization responses were measured in different

FIG. 2. (a) Schematic representation of the \( p \)-terphenyl crystal structure with the outer rings of the molecules (gray lines) and inner rings (black lines) \([20]\). (b) Two \( p \)-terphenyl molecules. The orientations of transition dipole moments in the unit cell frame are \( \phi \approx 33^\circ \) and \( \beta \approx 21^\circ \) \([20]\), with the same notations as in Fig. 1(a). (c) Euler set of angles \( (\theta, \phi, \psi) \) defining the unit cell frame (1, 2, 3) in the macroscopic frame (X, Y, Z). (d) A 3PEF image of a \( p \)-terphenyl nanocrystal, scale bar 200 nm. (e),(f) The 3PEF polarimetric responses of different \( p \)-terphenyl crystals: \( I_X \) (light gray markers) and \( I_Y \) (dark gray markers), fits (black lines) with \( (\theta, \phi, \psi) = (45^\circ, 90^\circ, 8^\circ) \) (e) and \( (67^\circ, 63^\circ, 85^\circ) \) (f). (g) A 3PEF polarimetric response observed in a disordered part of a \( p \)-terphenyl crystal.
FIG. 3. (a) A 3PEF image of a lysozyme crystal. Scale bar 10 μm. (b),(c) Typical 3PEF polarimetric responses of a crystal [the α (180°–360°) range is a copy of the (0°–180°) data]: \(I_y\) (light gray markers) and \(I_x\) (dark gray markers), fits (black lines) using (γ = 27°, β = 35°), (θ, φ, ψ) = (0°, 30°, 0°) (b) and (30°, 97°, 10°) (c), with notations of Figs. 1(a) and 2(c). (d) Calculated 3PEF polarimetric responses for different orientations of dipoles in the sample plane, illustrated schematically as arrows.

"p-terphenyl crystals of micrometric to nanometric sizes, taken from a powder [Fig. 2(d)]. The imaged crystals were of good optical quality and did not exhibit scattering. The majority of the observed \(I_x(\alpha)\) and \(I_y(\alpha)\) 3PEF polarization responses are of identical shapes, made of four lobes [Figs. 2(e) and 2(f)]. From the previous theoretical considerations, these results show that the measured structure is composed of at least four transition dipoles with various crystal orientations, and furthermore, that strong homo-FRET is present in the crystals (σ_A < 0.05). A fit of the polarization responses is based on Eq. (2), assuming the hypothesis of a homogeneous 3D coupling between the dipoles, as mentioned above, is valid, and based on five dipole-relative orientations known from the crystal structure. The fitting parameters are the Euler orientation angles (θ, φ, ψ) defining the crystal-unit cell orientation in the macroscopic frame [Fig. 2(c)]. These orientation parameters are obtained with a precision of about 5° and display good agreement of the model with the experimental data. In this analysis, we account for excitation polarization distortions from the optics in the microscope setup, as well as for the crystal birefringence, following methodologies previously published [21,22]. Nevertheless, some rare structures show very different \(I_x(\alpha)\) and \(I_y(\alpha)\) polarimetric shapes [Fig. 2(g)], which do not fit with this model. These responses are signatures of a local disorder at subdiffraction scales, which probably result from crystal grinding.

This methodology was further extended to protein crystals of lysozyme, a protein that contains six tryptophan residues with a minimum interdistance of 6 Å. The crystals belong to the P4_{1}2_{1}2_{1} space group (D_4 point group of tetragonal-trapezohedral class), with eight lysozyne molecules per unit cell [23]. Lysozyme crystals were prepared from 50 mg/ml of lysozyme from chicken egg whites, dissolved in a 0.1M sodium acetate buffer pH 4.5 and mixed 1:1 with 1M sodium chloride in the same buffer. The solution was placed in a quartz chamber. Crystals of good optical quality, with sizes from 5 μm to 1 mm grew within 12 h. Figure 3(a) shows a typical 3PEF microscopy image of a lysozyme crystal. Although protein crystals are much more fragile samples than molecular crystals (they have to be kept in an aqueous environment and are quite sensitive to photodamage) and their fluorescence signal is weaker overall, we could find reasonable intensity and integration times to measure polarimetric signatures from such crystals. Typical 3PEF polarimetric responses are depicted in Figs. 3(b) and 3(c). As was the case in p-terphenyl crystals, the \(I_x(\alpha)\) and \(I_y(\alpha)\) polarimetric patterns exhibit similar shapes, which also were observed in measurements performed on ten arbitrarily chosen crystals. In addition, the observed 3PEF polarimetric responses exhibit four-lobe patterns, showing that the structure most likely contains at least four transition dipole orientations arranged with a fourfold symmetry [Fig. 3(d)], consistent with its tetragonal symmetry. For this reason, in a first approximation we adopt a four-dipole model to fit the 3PEF polarization responses, even though the unit cell is known to possess 6 × 8 tryptophan molecules. Although only partial information on the crystal symmetry is retrieved from this measurement, it confirms the presence of efficient homo-FRET and the crystalline nature of the sample.

In this Letter, we have shown that polarization resolved 3PEF microscopy gives access to important information in molecular and protein crystals: whatever the analysis direction, there is a visible strong intermolecular energy transfer in the structures, illustrated by similar polarimetric shapes. Thus, this technique is a viable crystallinity diagnostic for such samples, which is particularly relevant for protein crystallization trials. Polarization resolved 3PEF microscopy is applicable to nanometric-size crystals, or to proteins lacking tryptophan residues (using fluorescence from the less-efficient tyrosine or phenylalanine amino acids), although this would require a more sensitive detection scheme.

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*alewit@pasteur.fr
\textsuperscript{1}sofie.brasselet@fresnel.fr
\textsuperscript{2}gafey@pasteur.fr
\textsuperscript{3}plauso@pasteur.fr