Orientation dependence in fluorescent energy transfer between Cy3 and Cy5 terminally attached to double-stranded nucleic acids

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We have found that the efficiency of fluorescence resonance energy transfer between Cy3 and Cy5 terminally attached to the 5′ ends of a DNA duplex is significantly affected by the relative orientation of the two fluorophores. The cyanine fluorophores are predominantly stacked on the ends of the helix in the manner of an additional base pair, and thus their relative orientation depends on the length of the helix. Observed fluorescence resonance energy transfer (FRET) efficiency depends on the length of the helix, as well as its helical periodicity. By changing the helical geometry from B form double-stranded DNA to A form hybrid RNA/DNA, a marked phase shift occurs in the modulation of FRET efficiency with helix length. Both curves are well explained by the standard geometry of B and A form helices. The observed modulation for both polymers is less than that calculated for a fully rigid attachment of the fluorophores. However, a model involving lateral mobility of the fluorophores on the ends of the helix explains the observed experimental data. This has been further modified to take account of a minor fraction of unstacked fluorophore observed by fluorescent lifetime measurements. Our data unequivocally establish that Förster transfer obeys the orientation dependence as expected for a dipole–dipole interaction.

cyanine fluorophores | FRET | kappa squared | single-molecule FRET

Fluorescence resonance energy transfer (FRET) has become widely used to report on distances over the macromolecular scale in biology (1), reviewed in refs. 2–4. The method is highly sensitive, and consequently has been widely exploited in single-molecule experiments in biological systems. Energy transfer results from dipolar coupling between the transition moments of two fluorophores, and the efficiency of the process (E_{FRET}) depends on the separation between the donor and acceptor fluorophores, raised to the sixth power. Although such data are frequently interpreted on the assumption of a simple relationship between E_{FRET} and distance, E_{FRET} should also depend on the relative orientation of the transition dipole vectors.

The orientation dependence is likely to be most significant where the fluorophores are constrained (5–9). This has been demonstrated experimentally by using a fluorophore that was terminally affixed to duplex DNA by two points of covalent attachment (10), thereby seriously constraining its motion. This situation is not typical of most FRET studies involving nucleic acids. Fluorophores are normally tethered by a single point of attachment, and in theory would be significantly less constrained. But if the fluorophores adopt a rigid manner of attachment to the helix, an orientational dependence could be observed.

Cy3 and Cy5 are a commonly used fluorophore pair, especially in single-molecule experiments. Our earlier NMR studies have shown that when these are attached to the 5′ termini of duplex DNA via a 3-carbon linker to the 5′-phosphate they are predominantly stacked onto the ends of the helix in the manner of an additional base pair (11, 26). This would provide a favorable situation in which the orientation dependence of FRET could be observed, and we have therefore studied a series of DNA and DNA-RNA hybrid duplexes to seek this effect. We find that E_{FRET} values reduce with duplex length, but also exhibit a modulation with twice the periodicity of the helices that is consistent with the anticipated orientation effect. Thus, it will be necessary to take fluorophore orientation into consideration when interpreting FRET data in terms of distances in some circumstances.

**Background Theory.** The variation of E_{FRET} with the separation between donor and acceptor fluorophores (R) is given by (12):

$$E_{\text{FRET}} = \frac{1}{1 + (R/R_0)^6}$$  \[1\]

where $R_0$ is the distance at which energy transfer is 50% efficient. It depends on the spectroscopic properties of the fluorophores and the medium, given by:

$$R_0^6 = \frac{0.529 \kappa^2 \Phi_D J(\lambda)}{N n^4}$$  \[2\]

where the units of $R_0$ and the wavelength $\lambda$ are centimeters. $\kappa^2$ describes the relative orientation of the fluorophores (see below). $\Phi_D$ is the quantum yield of the donor, $N$ is the Avogadro number, and $n$ is the index of refraction of the medium. $J(\lambda)$ is the spectral overlap integral, given by:

$$J(\lambda) = \frac{\int_0^\infty \phi_D(\lambda)e_d(\lambda)\lambda^4 d\lambda}{\int_0^\infty \phi_D(\lambda)d\lambda}$$  \[3\]

where $\phi_D(\lambda)$ is the spectral shape of donor emission and $e_d(\lambda)$ is the spectral shape of acceptor excitation (M⁻¹ cm⁻¹). We have

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measured the overlap integral for Cy3 and Cy5 terminally attached to dsDNA as \( J(\lambda) = 7.2 \times 10^{-13} \text{ M}^{-1} \text{ cm}^3 \).

The largest potential uncertainty in the extraction of distance information from \( E_{\text{FRET}} \) lies in the orientation term \( \kappa^2 \), given by:

\[
\kappa^2 = (\cos \Theta_T - 3 \cos \Theta_D \cos \Theta_A)^2
\]

where the angles \( \Theta_T \), \( \Theta_D \), and \( \Theta_A \) are defined in Fig. 1 A and B. This can take values between 0 and 4, or between 0 and 1 if the transition moments are constrained to parallel planes and perpendicular to the line joining them. If the fluorophores undergo isotropic reorientation in a time much shorter than the excited state lifetime of the donor, then \( \kappa^2 = 2/3 \) (6), and in most studies this is assumed to apply. Although this is a good approximation for freely mobile fluorophores like fluorescein that are terminally attached to double-stranded nucleic acids, it is rather less probable for cyanine dyes in this situation. By using NMR we have shown that Cy3 and Cy5 are predominantly stacked onto the end of dsDNA when coupled to the 5′-terminal phosphates via C3 linkers (11, 26) (Fig. 1 C and D). The transition moments for the \( \pi \) to \( \pi^* \) transitions lie in the plane of the indole rings close to the polyethylene linker (26), and are therefore directed in planes that are approximately parallel to each other. This situation approximates to \( \Theta_D = \Theta_A = 90^\circ \), and where \( \Theta_T \) depends on the length and geometry of the DNA helix and the angles between the terminal base pairs and the transition moments. This is illustrated in Fig. 1, on the assumption that the fluorophores are rigidly attached to the ends of the DNA. It can be seen that the efficiency of energy transfer is strongly modulated by the length of the helix in a periodic manner, falling close to zero twice per helix rotation when the transition moments are perpendicular (i.e., \( \cos \Theta_T = 0 \)). This “bouncing ball” type dependence is not significantly altered if the fluorophores are inclined relative to the terminal base pairs by 20°, as \( 3 \cos \Theta_D \cos \Theta_A \) is small and the behavior remains dominated by the variation in \( \Theta_T \). The variation of \( E_{\text{FRET}} \) with length with a constant value of \( \kappa^2 = 2/3 \) is also shown.

**Results and Discussion**

We have investigated the variation of FRET efficiency experimentally for a series of terminally labeled DNA duplexes of lengths 10–24 bp, thus covering more than one complete helical turn. Each species was generated by hybridizing extensively purified complementary strands with Cy3 or Cy5 separately attached to their 5′ termini via a three-carbon linker [see supporting information (SI) Fig. S1]. \( E_{\text{FRET}} \) was measured in both ensemble and single-molecule experiments.

**Modulation of Energy Transfer Efficiency in a DNA Duplex Series.** Each member of the series was studied in free solution under steady-state conditions, and \( E_{\text{FRET}} \) was calculated by using the acceptor normalization method (13). The resulting efficiencies are plotted as a function of helix length in Fig. 3 (filled circles). Overall, the \( E_{\text{FRET}} \) values decrease with helix length, yet there is a clear periodic modulation in phase with that anticipated for simple orientational dependence based on B form helical geometry (Fig. 2). Minima are observed at 13 and 18 bp, and maxima at 14 and 19 bp. The period is therefore \( \approx 5 \) bp, which is half the structural periodicity of a B form double helix; this is consistent with the anticipated orientational dependence of dipolar coupling. It should be noted that the observed modulation has twice the frequency of that observed where the interfluorophore separation varies with helix length because of off-axial positioning of freely mobile fluorophores (14).

The values of \( E_{\text{FRET}} \) for the DNA duplex series were measured...
by an alternative approach based on single-molecule methods. This was done for two main reasons. First, we were concerned that the ensemble results might be distorted by the presence of molecules in which Cy5 was not active. Second, the majority of experiments using the Cy3-Cy5 combination are performed with single-molecule methodology, and so this is directly relevant. $E_{\text{FRET}}$ was measured from single DNA molecules encapsulated in phospholipid vesicles (15) that were tethered to a quartz slide by using total internal reflection microscopy (16). This avoided any perturbation of DNA structure that might otherwise have arisen if there was a direct tether to the surface, and only molecules with active donor and acceptor fluorophores were analyzed. $E_{\text{FRET}}$ values of active species were calculated from the donor and acceptor wavelength channels, and histograms generated for many single molecules. The plot of $E_{\text{FRET}}$ vs. helix length (Fig. 3, open circles) is very similar to that generated from the ensemble data, with modulation of almost identical period and phase.

Thus, we observe the same modulation of FRET efficiency with helix length irrespective of the method of measurement. The period and phase of the modulation are fully consistent with the expected dependence on the relative orientation of the transition moments of the fluorophores, that is, the variation of $\kappa^2$.

A Dynamic Model Can Account for the Extent of Modulation. Although the positions of the maxima and minima of the experimental profiles are fully consistent with modulation due to fluorophore orientation, the peaks are clearly less sharply defined than those calculated for rigid fluorophores (Fig. 2), suggesting that the data are averaged by some dynamic process. Motions in which the fluorophores tilt away from their stacked position on the end of the DNA cannot explain this effect. We therefore simulated the data by using an alternative model in which the fluorophores are allowed to move laterally on the end of the helix, with a Gaussian distribution of probabilities of chosen half-width—this corresponds to a Boltzmann population of conformations where both fluorophores and the intervening DNA helix collectively act as a torsional spring. By using standard helical parameters for B form DNA with 10.5 bp per turn and a helical rise of 3.6 Å, together with mean angles between the terminal base pair and the transition moments of Cy3 and Cy5 of 32° and 30°, respectively (measured from the NMR structures), we generated simulations of the $E_{\text{FRET}}$ vs. helical length curves. We have assumed that the excitation and emission transition moments of Cy3 are parallel, which is supported by the high fundamental anisotropy of Cy3 (17). Setting a half-width at half-maximum (HWHM) of 55° provided excellent agreement with the shape of the experimental curve (Fig. 3), reproducing the maxima and minima in closely similar positions.

A Fraction of Cy3 Is Unstacked from the DNA. Although the lateral motion can account for the observed data very well, it does not provide a unique solution. A model in which one fraction of the fluorophores is stacked, whereas the remaining fluorophores are unstacked and freely mobile with $\kappa^2 = 2/3$, provided equally good agreement (see below). Although our NMR data indicate that most of the Cy3 will be stacked on the end of the DNA (11), a minor fraction that is unstacked would not be detected in these experiments. Leitus and coworkers (17) have provided time-resolved fluorescence data that indicate that there is a fraction of Cy3 that is free to rotate around its linker when attached to the 5′ terminus of double-stranded DNA. We therefore measured lifetime distributions for Cy3 attached to the 5′ terminus of DNA duplexes such that the fluorophore was in exactly the same environment as in the FRET experiments.

Singly Cy3-labeled duplexes of 16 and 22 bp were analyzed by using time-correlated photon counting, and the decay curves obtained fitted to a number of exponential functions (Fig. 4). For each sample our data were significantly better fitted by using three exponentials compared with two (see SI Materials and Table S1). Two species had relatively long lifetimes of 1.04 and 1.91 ns, with fractional intensities of 61.6% and 21.4%, respectively for the 16-bp duplex, whereas the third species had a lifetime of 390 ps and an intensity of 16.9% (Table 1). Identical results were obtained for the 22-bp duplex within experimental error. We assign the short-lifetime species to unstacked Cy3, where the excited state is rapidly relaxed by cis-trans photoisomerization occurring in the polyethylene linker (18). The fluorescent lifetime becomes longer if rotation about the polyethylene linkage is prevented, for example, by steric constraints in Cy3B (17). Thus, stacking may also prevent isomerization and account for the species we see with longer lifetimes. We suggest that the species with the longest lifetime has Cy3 stacked onto the end of the helix in a manner similar to that we have observed by NMR (11), because its lifetime is very close to the 2 ns expected for a Cy3 species that is unable to photoisomerize, calculated from the radiative fluorescence rate for Cy3 of $5 \times 10^8$ s$^{-1}$ (17). The intermediate lifetime probably arises from Cy3 in a series of environments in which rotation about the polyethylene linkage of the fluorophore is constrained but not prevented. These are unlikely to be freely mobile, because such a high proportion of unstacked fluorophore is not consistent with the NMR data. However, the lateral motion could partially expose the fluorophore such that some segmental rotation within the polyethylene linker becomes possible, shortening the fluorescent lifetime.

Assuming that the dynamic properties of the chemically similar Cy5 are comparable to Cy3, up to 31% of the fluorescent
emission could be due to molecules in which one or both fluorophores are in an unstacked conformation. We therefore simulated our data assuming that 31% of the molecules had freely mobile fluorophores such that $\kappa^2 = 2/3$ (Fig. 4B). This provides an equally good fit to the experimental data (both ensemble and steady-state) compared with the fully stacked model, giving a HWHM = 42° for the fluorophores remaining stacked on the helix. However, simulations (data not shown) show that using a free fraction of fluorophore plus a stacked fraction with no lateral flexibility cannot explain the data. First, the minima and maxima are no longer in the correct positions. Second, we require a free fraction of $\approx$75% to approximate the experimental data, and this is in plain contradiction with our earlier NMR data (11).

**An Altered Periodicity of Modulation in a DNA-RNA Hybrid Duplex Series.** The modulation of $E_{\text{FRET}}$ with twice the frequency of the DNA helix provides strong evidence for the orientation dependence of the dipolar coupling. And, this suggests a further test of the model. On the basis of this model we expect that an A form helix should produce a similar modulation, but with an altered period and phase because this helical conformation is less tightly wound than the B form helix of double-stranded DNA. We therefore tested the prediction by the construction of a new series of terminally Cy3-Cy5-labeled duplex species with one DNA and one RNA strand. These hybrid duplexes should adopt an A form helix (19–21). FRET efficiency was measured by using both steady-state ensemble spectroscopy and single encapsulated molecules by TIR microscopy as before. The data are presented in Fig. 5, with the data for the DNA duplexes reproduced in gray for comparison. The profiles have the same appearance as those for the DNA duplexes, with a clear modulation superimposed onto a generally reducing $E_{\text{FRET}}$ as duplex length increases. However, the modulation is clearly not in phase with the DNA data, with maxima observed at 11, 17, and 22 bp. The efficiency of energy transfer is modulated with twice the periodicity of a helix corresponding to $\approx$12 bp per turn, that is, in good agreement with that expected for an A form helix.

We also carried out time-resolved fluorescence lifetime measurements of Cy3 attached to DNA/RNA duplexes of 16 and 22 bp in length (see SI Materials, Table S1, and Fig. S2). As with the DNA, the data were best fitted by using three species, with lifetimes (amplitudes) of 330 ps (6.1%), 990 ps (46.4%), and 1.84 ns (47.5%) (average for the 16 and 22 bp species). As with the DNA, we attribute the short-lifetime species to unstacked Cy3 molecules, and the remaining species to stacked fluorophores. These data indicate that the Cy3 is stacked more fully on the A form helix compared with the DNA duplex.

On the basis of these results we simulated our FRET efficiencies as a function of DNA/RNA duplex length based on an A form helix with 12 bp per turn and a rise of 3 Å and an unstacked fraction of fluorophores of 12% with $\kappa^2 = 2/3$ (Fig. 5).

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**Table 1. Fluorescent lifetime data for 5-Cy3-labeled 16-bp DNA and RNA/DNA duplexes**

<table>
<thead>
<tr>
<th></th>
<th>$\tau$, ns</th>
<th>$\alpha$</th>
<th>$f$, %</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA 16 bp</td>
<td>0.39</td>
<td>0.018</td>
<td>16.9</td>
<td>1.062</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>0.025</td>
<td>61.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.91</td>
<td>0.005</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td>RNA/DNA 16 bp</td>
<td>0.31</td>
<td>0.009</td>
<td>5.3</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>0.96</td>
<td>0.025</td>
<td>43.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.77</td>
<td>0.016</td>
<td>51.0</td>
<td></td>
</tr>
</tbody>
</table>

$\tau$, $\alpha$, and $f$ are the fluorescent lifetime, lifetime amplitude, and fractional intensity for each component, and $\chi^2$ is the chi-squared statistic.
Good agreement with the experimental data was obtained by inclusion of lateral fluorophore mobility with HWHM = 42° for the stacked fluorophores, and a refractive index \( n = 1.33 \). To obtain the best absolute agreement, a value of fluorescence quantum yield of 0.35 was used for the DNA-RNA duplexes, consistent with the greater proportion of the long-lifetime species.

**Conclusions**

The observed modulation of the distance dependence of FRET for the DNA and hybrid DNA-RNA series, and the agreement with simulations based on B and A form helices, provide strong evidence for the orientational dependence (i.e., \( \kappa^2 \)) of energy transfer efficiency by using the commonly employed Cy3-Cy5 donor–acceptor pair. Our experiments unequivocally establish that Förster transfer obeys the orientation dependence as expected for a dipole–dipole interaction. In many situations a simple inverse-distance interpretation of FRET efficiency will provide an adequate qualitative interpretation, but our data show that the common assumption that FRET is a monotonous function of distance can actually fail under certain circumstances. For the extraction of precise distance information, it will be necessary to take account of the orientation dependence. From our data we calculate that the assumption that \( \kappa^2 = 2/3 \) could result in an error of up to 12 Å in distance estimation in some circumstances, notably when the transition moments are close to perpendicular. The discrepancy might be reduced if significant flexibility can be introduced into the linker connecting the two fluorophores. For example, changing the length and characteristics of the covalent tether might result in a flexible fluorophore, although this cannot be assumed a priori. If the fluorophores remain stacked on the helix, the orientation effect could lead to misassignment of states in single-molecule experiments if the assignment is made based only on the FRET efficiencies, especially in more complex systems with multiple states. Therefore, additional control experiments should be performed to provide independent support for the assignments. On the positive side, the orientation effects in FRET could be a valuable tool in structural biology. A full understanding of the orientation dependence could greatly extend the use of FRET measurements to provide both accurate distance and angular information.

**Materials and Methods**

**Synthesis and Preparation of Duplex Species.** Deoxyribooligonucleotides were synthesized by using standard phosphoramidite chemistry, and ribooligonucleotides were synthesized by using 2′-β-DMS ribonucleoside β-cyanoethyl phosphoramidites (22), as described in ref. 23. Cy3 and Cy5 were added to 5′ termini as phosphoramidites at the end of synthesis as required. Fully depurinated oligonucleotides were purified by electrophoresis in 20% polyacrylamide gels containing 7 M urea, and recovered by electroelution. The cyano-conjugated strands were further purified by reversed-phase chromatography with a C18 column eluted with a gradient of acetonitrile in 100 mM triethylammonium acetate. Duplex species were assembled by mixing stoichiometric quantities of purified DNA (Cy3 or Cy5 labeled) or RNA (Cy3 labeled) in 90 mM Tris-borate (pH 8.3), 25 mM NaCl, cooling slowly from 95°C to 4°C. Duplexes were then purified by electrophoresis in 20% polyacrylamide under non-denaturing conditions in 90 mM Tris-borate (pH 8.3), 25 mM NaCl. Gel fragments containing the required duplexes were excised, and the double-stranded nucleic acids were recovered by electroelution. The purified DNA or DNA/RNA species were dissolved in 90 mM Tris-borate (pH 8.3) and absorption spectra recorded from 220 to 800 nm by using a Cary 1E UV-visible spectrophotometer. The full sequences of all of the duplex species used in this study are listed in **SI Materials**.

**Steady-State Ensemble Fluorimetry.** Fluorescence emission spectra were recorded at 4°C by using an SLM-Aminco 8100 fluorimeter in 90 mM Tris-borate (pH 8.3). Spectra were corrected for lamp fluctuations and instrumental variations, and polarization artifacts were avoided by crossing excitation and emission polarizers at 54.7°. \( E_{\text{FRET}} \) was measured with the acceptor normalization method (13) by using emission spectra (550–720 nm) excited at 535 and 600 nm.

**Single-Molecule Analysis of Encapsulated Duplex Species.** Single duplex molecules were studied trapped within phospholipid vesicles in 10 mM TrisHCl (pH 8.0), 50 mM NaCl. Individual DNA or RNA/DNA duplex species were encapsulated in phospholipid vesicles comprising a 100:1 mixture of either L-α-phosphatidylcholine or 1,2-dimyristoyl-sn-glycero-3-phosphocholine with...
1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (Avanti Polar Lipids) by repeated extrusion through a polycarbonate membrane containing 200-nm pores (Osmonics) (24) as described (15). This generated 200-nm-diameter vesicles, with a 1:1 molar ratio of duplex to vesicles. The vesicles were conjugated via NeutrAvidin (Pierce) to biotin-functionalized polyethylene glycol (PEG)-coated quartz surfaces. Encapsulated molecules were excited at 532 nm by prism-based total internal reflection (16) microscope at 12°C. Imaging of the surface at Cy3 and Cy5 emission wavelengths were obtained by using an Andor intensified CCD camera, with an integration time of 100 ms for durations of 2–3 s. Dual-view design of the emission pathway allowed us to image the Cy3 and Cy5 signals simultaneously. \( E_{\text{RET}} \) values were calculated from averaged Cy3 and Cy5 intensities over 300- to 500-ms periods for each molecule in the image. Data were corrected for crosstalk between Cy3 and Cy5 channels, backreflection from the dichroic mirror surface in the dual-view emission pathway, and the background (see SI Materials). Histograms of \( E_{\text{RET}} \) values from 5,000 to 32,000 molecules are plotted in Fig. S3. Each histogram contained two major peaks; one at \( E_{\text{RET}} = 0 \) corresponding to duplexes with active Cy3 only, and another at higher \( E_{\text{RET}} \) resulting from duplex molecules with an active Cy3-Cy5 pair. However, for some constructs, an additional, broad peak was observed at intermediate \( E_{\text{RET}} \) values because of some vesicles containing multiple DNA molecules with at least one photobleached Cy5. In such cases, we fit the data with three Gaussian peaks to separate the contribution of the important species (see SI Materials and Fig. S4). Errors on the reported \( E_{\text{RET}} \) values represent the standard deviation of randomly sampled subsets of the data, each comprising \( -1,000 \) molecules.

**Time-Resolved Fluorimetry.** Time-resolved fluorescence intensity measurements were performed by time-correlated single-photon counting (TCSPC) by using an FL 920 spectrometer (Edinburgh Instruments) (25). The excitation source was a titanium sapphire laser (Coherent) with 200-fs pulse duration operated at 76 MHz. Its fundamental output was sent through a pulse picker (Coherent 9200) and a harmonic generator (Coherent 5-050) to obtain 450-nm pulses at 4.7 MHz. The excitation beam was attenuated as needed to avoid pile-up effects. Fluorescence emission was detected by using a monochromator and a cooled microchannel plate photomultiplier tube (Hamamatsu C4878). The instrument response function, measured by scattering the excitation beam from a dilute suspension of colloidal silica (Ludox), was less than 100 ps FWHM. Fluorescence decay curves were recorded on a time scale of 20 ns, resolved into 4,096 channels, to a total of 10,000 counts in the peak channel. Decay curves were analyzed by using a standard iterative reconvolution method in the F900 (Edinburgh Instruments) software packages, on the basis of a multieponential decay function. The quality of fit was judged on the basis of the reduced \( \chi^2 \) statistic, and the randomness of residuals.

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Three-Dimensional Super-Resolution Imaging by Stochastic Optical Reconstruction Microscopy

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Recent advances in far-field fluorescence microscopy have led to substantial improvements in image resolution, achieving a near-molecular resolution of 20 to 30 nanometers in the two lateral dimensions. Three-dimensional (3D) nanoscale-resolution imaging, however, remains a challenge. We demonstrated 3D stochastic optical reconstruction microscopy (STORM) by using optical astigmatism to determine both axial and lateral positions of individual fluorophores with nanometer accuracy. Iterative, stochastic activation of photoswitchable probes enables high-precision 3D localization of each probe, and thus the construction of a 3D image, without scanning the sample. Using this approach, we achieved an image resolution of 20 to 30 nanometers in the lateral dimensions and 50 to 60 nanometers in the axial dimension. This development allowed us to resolve the 3D morphology of nanoscopic cellular structures.

Far-field optical microscopy offers three-dimensional (3D) imaging of biological specimens with minimal perturbation and biomolecular specificity when combined with fluorescent labeling. These advantages make fluorescence microscopy one of the most widely used imaging methods in biology. The diffraction barrier, however, limits the imaging resolution of conventional light microscopy to 200 to 300 nm in the lateral dimensions, leaving many intracellular organelles and molecular structures unresolved. Recently, the diffraction limit has been surpassed and lateral imaging resolutions of 20 to 50 nm have been achieved by several “super-resolution” far-field microscopy techniques, including stimulated emission depletion (STED) and its related RESOLFT (reversible saturable optical nonlinear fluorescence transitions) microscopy (1, 2), saturated structured illumination microscopy (SSIM) (3); stochastic optical reconstruction microscopy (STORM) (4, 5); photoactivated localization microscopy (PALM) (6, 7); and other methods using similar principles (8–10).

Although these techniques have improved 2D image resolution, most organelles and cellular structures cannot be resolved without high-resolution imaging in all three dimensions. Three-dimensional fluorescence imaging is most commonly performed using confocal or multiphoton microscopy, the axial resolution of which is typically in the range of 500 to 800 nm (11, 12). The axial imaging resolution can be improved to roughly 100 nm by 4Pi and 1M microscopy (13–15). Furthermore, an axial resolution as high as 30 to 50 nm has been obtained with STED along the axial direction using the 4Pi illumination geometry, but the same imaging scheme does not provide super resolution in the lateral dimensions (1).

Here, we demonstrate 3D STORM imaging with a spatial resolution that is 10 times better than the diffraction limit in all three dimensions without invoking sample or opticalbeam scanning. STORM and PALM rely on single-molecule detection (16) and exploit the photoswitchable nature of certain fluorophores to temporally separate the otherwise spatially overlapping images of numerous molecules, thereby allowing the high-precision localization of individual molecules (4–7, 9). Limited only by the number of photons detected (17), localization accuracies as high as 1 nm can be achieved in the lateral dimensions for a single fluorescent dye at ambient conditions (18).

Not only can the lateral position of a particle be determined from the centroid of its image (19, 20), the shape of the image also contains information about the particle’s axial (z) position. Nanoscale localization accuracy has been achieved in the z dimension by introducing defocusing (21–24) or astigmatism (25, 26) into the image, without substantially compromising the lateral positioning capability.

In this work, we used the astigmatism imaging method to achieve 3D STORM imaging. To this end, a weak cylindrical lens was introduced into the imaging path to create two slightly different focal planes for the x and y directions (Fig. 1A) (25, 26). As a result, the ellipticity and orientation of a fluorophore’s image varied as its position changed in z (Fig. 1A). When the fluorophore was in the average focal plane [approximately halfway between the x and y focal planes where the point spread function (PSF) has equal widths in the x and y directions], the image appeared round; when the fluorophore was above the average focal plane, its image was more focused in the y direction than in the x direction and thus appeared elliptoidal with its long axis along x; conversely, when the fluorophore was below the average focal plane, the image appeared elliptoidal with its long axis along y. By fitting the image with a 2D elliptical Gaussian function, we obtained the x and y coordinates of the peak position as well as the peak widths w_x and w_y, which in turn allowed the z coordinate of the fluorophore to be unambiguously determined.

To experimentally generate a calibration curve of w_x and w_y as a function of z, we immobilized Alexa 647–labeled streptavidin molecules or quantum dots on a glass surface and imaged individual molecules to determine the w_x and w_y values as the sample was scanned in z (Fig. 1B). In 3D STORM analysis, the z coordinate of each photoactivated fluorophore was then determined by comparing the measured w_x and w_y values of its image with the calibration curves. In addition, for samples immersed in aqueous solution on a glass substrate, all z localizations were rescued by a factor...
of 0.79 to account for the refractive index mismatch between glass and water [see (27) for a detailed description of the analysis procedures].

The 3D resolution of STORM is limited by the accuracy with which individual photoactivated fluorophores can be localized in all three dimensions during a switching cycle. We recently discovered a family of photoswitchable cyanine dyes (Cy3, Cy5.5, Cy7, and Alexa 647) that can be reversibly cycled between a fluorescent and a dark state by light of different wavelengths. The reactivation efficiency of these photoswitchable “reporters” depends critically on the proximity of an “activator” dye, which can be any one of a variety of dye molecules (e.g., Cy3, Cy2, Alexa 405) (5, 28). We used Cy3 and Alexa 647 as the activator and reporter pair to perform 3D STORM imaging. A red laser (657 nm) was used to image Alexa 647 molecules and deactivate them to the dark state; a green laser (532 nm) was used to reactivate Alexa 647

**Fig. 1.** The scheme of 3D STORM. (A) Three-dimensional localization of individual fluorophores. The simplified optical diagram illustrates the principle of determining the z coordinate of a fluorescent object from the ellipticity of its image by introducing a cylindrical lens into the imaging path. The right panel shows images of a fluorophore at various z positions. EMCCD, electron-multiplying charge-coupled device. (B) Calibration curve of image widths \( w_x \) and \( w_y \) as a function of \( z \) obtained from single Alexa 647 molecules. Each data point represents the average value obtained from six molecules. The data were fit to a de-focusing function (red curve) as described in (27). (C) Three-dimensional localization distribution of single molecules. Each molecule gives a cluster of localizations due to repetitive activation of the same molecule. Localizations from 145 clusters were aligned by their center of mass to generate the overall 3D presentation of the localization distribution (left panel). Histograms of the distribution in \( x \), \( y \), and \( z \) (right panels) were fit to a Gaussian function, yielding standard deviations of 9 nm in \( x \), 11 nm in \( y \), and 22 nm in \( z \).

**Fig. 2.** Three-dimensional STORM imaging of microtubules in a cell. (A) Conventional indirect immunofluorescence image of microtubules in a large area of a B5-C-1 cell. (B) The 3D STORM image of the same area, with the z-position information color-coded according to the color scale bar. Each localization is depicted in the STORM image as a Gaussian peak, the width of which is determined by the number of photons detected (5). (C to E) The \( x-y \), \( x-z \), and \( y-z \) cross sections of a small region of the cell outlined by the white box in (B), showing five microtubule filaments. Movie S1 shows the 3D representation of this region, with the viewing angle rotated to show different perspectives (27). (F) The \( z \) profile of two microtubules crossing in the \( x-y \) projection but separated by 102 nm in \( z \), from a region indicated by the arrow in (B). The histogram shows the distribution of \( z \) coordinates of the localizations, fit to two Gaussians with identical widths (FWHM = 66 nm) and a separation of 102 nm (red curve). The apparent width of 66 nm agrees quantitatively with the convolution of our imaging resolution in \( z \) (represented by a Gaussian function with FWHM of 55 nm) and the previously measured width of antibody-coated microtubules (represented by a uniform distribution with a width of 56 nm) (5).
in a Cy3-dependent manner (5, 28). Each activator-reporter pair could be cycled on and off hundreds of times before permanent photobleaching occurred. An average of 6000 photons were detected per switching cycle by means of objective-type total internal reflection fluorescence or epifluorescence imaging geometry. This reversible switching behavior provided an internal control to measure the localization accuracy. To this end, we immobilized streptavidin molecules doubly labeled with Cy3 and Alexa 647 on a glass surface (27). The molecules were then switched on and off for multiple cycles, and their x, y, and z coordinates were determined for each switching cycle (27). This procedure resulted in a cluster of localizations for each molecule (Fig. 1C). The standard deviations of the localization distribution obtained within 100 nm of the average focal plane were 9 nm in x, 11 nm in y, and 22 nm in z, and the corresponding full width at half maximum (FWHM) values were 21 nm, 26 nm, and 52 nm, providing a quantitative measure of the localization accuracy in 3D (Fig. 1C). The localization accuracies in the two lateral dimensions were similar to our previous 2D STORM resolution obtained without the cylindrical lens (5). The localization accuracy in z was approximately twice the localization accuracy measured in x and y. Because the image width increases as the fluorophore moves away from the focal plane, the localization accuracy decreases with increasing absolute values of z, especially in the lateral dimensions. Therefore, we typically chose a z imaging depth of about 600 nm near the focal plane, within which the lateral and axial localization accuracies varied by factors of <1.6 and <1.3, respectively, relative to the values obtained at the average focal plane. The imaging depth may, however, be increased by the use of z scanning in future experiments.

As an initial test of 3D STORM, we imaged a model bead sample prepared by immobilizing 200-nm biotinylated polystyrene beads on a glass surface and then incubating the sample with Cy3- and Alexa 647–labeled streptavidin to coat the beads with photoswitchable probes (27). Three-dimensional STORM images of the beads were obtained by iterative, stochastic activation of sparse subsets of optically resolvable Alexa 647 molecules, allowing the x, y, and z coordinates of individual molecules to be determined. Over the course of multiple activation cycles, the positions of numerous fluorophores were determined and used to construct a full 3D image (27). The projections of the bead images appeared approximately spherical when viewed along all three directions, with average diameters of 210 ± 16, 225 ± 25, and 228 ± 25 nm in x, y, and z, respectively (fig. S1) (27), indicating accurate localization in all three dimensions. Because the image of each fluorophore simultaneously encodes its x, y, and z coordinates, no additional time was required to localize each molecule in 3D STORM as compared with 2D STORM imaging.

Applying 3D STORM to cell imaging, we next performed indirect immunofluorescence imaging of the microtubule network in green monkey kidney epithelial (BS-C-1) cells. Cells were immunostained with primary antibodies and then with secondary antibodies doubly labeled with Cy3 and Alexa 647 (27). The 3D STORM image not only showed a substantial improvement in resolution over the conventional wide-field fluorescence image (Fig. 2, A and B), but also provided the z-dimension information (color-coded in Fig. 2B) that was not available in the conventional image. Multiple layers of microtubule filaments were clearly visible in the

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**Fig. 3.** Three-dimensional STORM imaging of clathrin-coated pits in a cell. (A) Conventional direct immunofluorescence image of clathrin in a region of a BS-C-1 cell. (B) The 2D STORM image of the same area, with all localizations at different z positions included. (C) An x–y cross section (50 nm thick in z) of the same area, showing the ring-like structure of the periphery of the CCPs at the plasma membrane. (D and E) Magnified view of two nearby CCPs in 2D STORM (D) and their x–y cross section (100 nm thick) in the 3D image (E). (F to H) Serial x–y cross sections (each 50 nm thick in z) (F) and x–z cross sections (each 50 nm thick in y) (G) of a CCP, and an x–y and x–z cross section presented in 3D perspective (H), showing the half-spherical cage-like structure of the pit.
x-y, x-z, and y-z cross sections of the cell (Fig. 2, C to E, and movie S1) (27).

To characterize our cell imaging resolution more quantitatively, we identified point-like objects in the cell that appeared as small clusters of localizations away from any discernible microtubule filaments. These clusters likely represent individual antibodies nonspecifically attached to the cell. The FWHM values of these clusters, which were randomly chosen over the entire measured z-range of the cell, were 22 nm in x, 28 nm in y, and 55 nm in z (Fig. S2) (27), similar to those determined for individual molecules immobilized on a glass surface (compare fig. S2 with Fig. 1C). Two microtubule filaments separated by 100 nm in z appeared well separated in the 3D STORM image (Fig. 2F). The apparent width of the microtubule filaments in the z dimension was 66 nm, slightly larger than our intrinsic imaging resolution in z and in quantitative agreement with the convolution of the imaging resolution and the independently measured width of the antibody-coated microtubule (Fig. 2F). Because the effective resolution is determined by a combination of the intrinsic imaging resolution (as characterized above) and the size of the labels (e.g., antibodies), improved resolution may be achieved by using direct immunofluorescence to remove one layer of antibody labeling, as we show in the next example, or by using Fab fragments or genetically encoded peptide tags (29, 30) in place of antibodies.

Finally, to demonstrate that 3D STORM can resolve the 3D morphology of nanoscopic structures in cells, we imaged clathrin-coated pits (CCPs) in BS-C-1 cells. CCPs are spherical cage-like structures about 150 to 200 nm in size, assembled from clathrin and cofactors on the cytoplasmic side of the cell membrane to facilitate endocytosis (37). To image CCPs, we adopted a direct immunofluorescence scheme using primary antibodies against clathrin doubly labeled with Cy3 and Alexa 647 (27). When imaged by conventional fluorescence microscopy, all CCPs appeared as nearly diffraction-limited spots with no discernible structure (Fig. 3A). In 2D STORM images in which the z-dimension information was discarded, the round shape of CCPs was clearly seen (Fig. 3, B and D). The size distribution of CCPs measured from the 2D projection image, 180 ± 40 nm, agrees quantitatively with the size distribution determined using electron microscopy (EM) (32). Including the z-dimension information allowed us to clearly visualize the 3D structure of the pits (Fig. 3, C and E to H). Figures 3C and 3E show the x-y cross sections of the image, taken from a region near the opening of the pits at the cell surface. The circular ring-like structure of the pit periphery was unambiguously resolved. Consecutive x-y and x-z cross sections of the pits (Fig. 3, F to H) clearly revealed the half-spherical cage-like morphology of these nanoscopic structures that was not observable in the 2D images. These experiments demonstrate the ability of 3D STORM to resolve nanoscopic features of cellular structures with molecular specificity under ambient conditions.

References and Notes
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Supporting Online Material
www.sciencemag.org/cgi/content/full/11253529/DC1
Materials and Methods
Figs. S1 and S2
Movie S1
References
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An Association Between the Kinship and Fertility of Human Couples

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Previous studies have reported that related human couples tend to produce more children than unrelated couples but have been unable to determine whether this difference is biological or stems from socioeconomic variables. Our results, drawn from all known couples of the Icelandic population born between 1800 and 1965, show a significant positive association between kinship and fertility, with the greatest reproductive success observed for couples related at the level of third and fourth cousins. Owing to the relative socioeconomic homogeneity of Icelanders, and the observation of highly significant differences in the fertility of couples separated by very fine intervals of kinship, we conclude that this association is likely to have a biological basis.

There has been long-standing uncertainty about the impact of kinship or consanguinity between spouses on the total number of offspring they produce (completed fertility). Consanguineous unions among humans increase the probability of a zygote receiving the same deleterious recessive alleles from both parents, with a possible adverse effect on fertility through an increased rate of miscarriage, infant mortality, and morbidity (1–3). Conversely, consanguineous unions may confer greater completed fertility through earlier age at marriage, as well as the socioeconomic advantages associated with preserving land and wealth within extended families (4, 5). In other species, lower fitness has been observed in offspring of distantly related individuals, which appears to be a result of the breakdown of coadapted gene complexes (6).

Previous studies examining the relationship between kinship and fertility in humans have focused on relatively close relationships between couples, rarely evaluating relationships more distant than second cousins (who share two great-grandparents) (4). Such studies have tended to be

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