Background: We report on the potential DNA binding modes and spectral characteristics of the cell-permeant far red fluorescent DNA dye, DRAQ5, in solution and bound within intact cells. Our aim was to determine the constraints for its use in flow cytometry and bioimaging.

Methods: Solution characteristics and quantum yields were determined by spectroscopy. DRAQ5 binding to nuclear DNA was analyzed using fluorescence quenching of Hoechst 33342 dye, emission profiling by flow cytometry, and spectral confocal laser scanning microscopy of the complex DRAQ5 emission spectrum. Cell cycle profiling utilized an EGFP–cyclin B1 reporter as an independent marker of cell age. Molecular modeling was used to explore the modes of DNA binding.

Results: DRAQ5 showed a low quantum yield in solution and a spectral shift upon DNA binding, but no significant fluorescence enhancement. DRAQ5 caused a reduction in the fluorescence intensity of Hoechst 33342 in live cells prelabeled with the UV excitable dye, consistent with molecular modeling that suggests AT preference and an engagement of the minor groove. In vivo spectral analysis of DRAQ5 demonstrated shifts to longer wavelengths upon binding with DNA. Analysis of spectral windows of the dual emission peaks at 681 and 707 nm in cells showed that cell cycle compartment recognition was independent of the far red–near IR emission wavelengths monitored.

Conclusions: The study provides new clues to modes of DNA binding of the modified anthraquinone molecule in vivo, and its AT base-pair selectivity. The combination of low quantum yield but high DNA affinity explains the favorable signal-to-noise profile of DRAQ5-nuclear fluorescence. The robust nature of cell cycle reporting using DRAQ5, even when restricted spectral windows are selected, facilitates the analysis of encroaching spectral emissions from other fluorescent reporters, including GFP-tagged proteins.

Key terms: DRAQ5; spectral imaging; EGFP-cyclin B1; DNA-binding; cell cycle

The chromophores of DNA-binding agents have been exploited extensively in spectroscopic and live cell cytometric studies to probe features of the nuclear compartment and to study the dynamics of ligand binding. Advances in microtechnologies for the handling of cells combined with widespread adoption of green fluorescent protein (GFP)-based reporters and the availability of red-light emitting semiconductor lasers have further raised interest in rapidly staining, low IR-emitting DNA fluor for event recognition in biochip devices (1). Tracking DNA-ligand interactions in live cells also facilitates the design of anticancer agents, since many drugs are naturally fluorescent. Reversible DNA-binding agents can affect DNA metabolism and modify the discrete function of specific pro-
tein targets for molecular therapeutics, as shown by the anthraquinone-mediated inhibition of DNA topoisomerase II function (3) and the anthracycline-mediated blocking of mismatch repair (3). Critically, both the development of novel drugs with DNA binding properties (i.e. DNA-affinic) and target specificity, and molecular probes with desired chromophore performance must address the sequence preferences of ligands and the modes of DNA interaction. Further, the behavior of ligands within the intracellular chromatin complex could be affected by the condensation/decondensation cycles of the cell cycle. Fluorescence methods can be used to inform predictive systems models of the cellular environment and kinetics of nuclear targeting (4). Such considerations provide a clear impetus for understanding the information content of fluorescence signatures from single cells. Here, we have undertaken spectral analysis of the behavior of the novel DNA binding agent, DRAQ5 (5,6), in intact cells to address binding modes in live cells, nuclear feature resolution, and cell cycle profiling, with specific reference to the application of GFP-based reporters.

In general, if agents possess sequence-specific binding to double-stranded DNA, then there are two common reversible (noncovalent) binding modes—intercalation between base pairs or groove binding. However, molecular modeling and in vitro studies on DNA–ligand complexes often reveal the potential for multiple binding modes. Anthracyclines comprise an important class of noncovalent binding anticancer antibiotics, with a chromophore that contains the iron-chelating hydroxyquinone structure (7). 1,4-Dihydroxyanthraquinone is the smallest molecule to display the chromophore characteristics of the anthracycline antibiotics, with a broad UV–visible absorbance spectrum in solution (360–515 nm). The absorbance spectrum in solution (360–515 nm). The absorbance spectrum of 1,4-dihydroxyanthraquinone/DNA complex shifts the spectrum to the lower energy range (longer wavelength) of the absorbance spectrum, consistent with red-light excitation of the chromophore (8). The visible absorption band of the parent molecule can be assigned to a single electronic state, with a reduced dipole moment in the excited state (8). Previous molecular modeling studies have suggested that anthraquinone derivatives, substituted at the 1,4- and 1,8-o-NH(CH2)2NH(CH2CH3)2 side chain, intercalate with DNA, with both substituents in the same groove (classical intercalation), while the similarly substituted 1,5-derivative (DRAQ5) intercalates in a threading mode, with a side chain in each groove (9). DRAQ5 has high DNA binding affinity, while its anthraquinone chromophore is only weakly fluorescent (5,10). Absorbance or fluorescence wavelength shifts and changes in quantum yield can signal complexation of the ligand with DNA (11), and we have studied the change of DRAQ5 fluorescence quantum yield to investigate the extent of fluorescence enhancement upon binding to DNA and the nature of the interaction (11,12).

The minor groove binding bisbenzimidye dyes, Hoechst 33258 and Hoechst 33342, are cell-permeant DNA dyes that demonstrate fluorescent enhancement upon binding to DNA. These dyes are UV-excitable with excitation/emission maxima at ~350/460 nm. Additionally their fluorescence undergo a subtle violet-to-red shift as DNA binding proceeds (13,14). The spectral shift suggests multiple binding modes dependent on dye:base-pair ratios. These dyes appear to show a wide spectrum of AT-sequence selectivity, with a capacity to displace some DNA intercalators (15), and can therefore act as intracellular reporters for nuclear binding sites. Here, we describe in a general manner the reduction of Hoechst 33342-DNA fluorescence in the presence of DRAQ5 as a “quenching” effect, although the molecular interactions that give rise to this process are not specified. This provides a pragmatic route for ascertaining the potential overlap of target sites with less well-understood DNA-affinic agents, such as the anthraquinones. Here the Hoechst dye is used as a reference. Our aim was to understand the spectral (absorption and emission) characteristics and components of the DRAQ5 single state fluorescence profile while bound to nuclear DNA. We demonstrate a significant overlap of binding sites for Hoechst 33342 and DRAQ5 and consider this within a molecular modeling study. We further report that high-resolution spectral imaging of DRAQ5 bound to nuclear chromatin reveals an emission spectrum with distinct double peaks—potentially distinguishing unbound and intercalated probe. Accordingly, we have assessed the implications of the spectral analysis study for the performance of DRAQ5 in cell cycle profiling studies and for emission wavelength selection, when combined with GFP-based reporters.

**MATERIALS AND METHODS**

**Cell Culture**

The transformed human osteosarcoma cell line (U-2 OS) expressing GFP–cyclin B1, was cultured under G418 selection, as previously reported (16), in McCoy’s 5a medium (Sigma-Aldrich, Dorset, UK), supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, and antibiotics, and incubated at 37°C in an atmosphere of 5% CO2 in air. For imaging experiments, cells were grown at a density of 1 × 10^6 cells/ml as a monolayer in two well Nunc culture dishes or as asynchronously growing suspension cultures of HL60 cells were diluted to 2.5 to 4 × 10^6 cells/ml at 2 h prior to drug treatment. Cells were treated with Hoechst 33342 or DRAQ5 or both, as described later. All cell concentrations were determined using a Coulter particle counter (Beckman Coulter, High Wycombe, UK). Cell cycle distribution was determined using an algorithm for the normal distribution of fluorescence intensity (FI) profiles for fluorochrome-stained G1 and G2 cells (17).

**Reagent Preparation and Treatments**

DRAQ5 was supplied by Biostatus (Leicestershire, UK), as an acidified aqueous stock solution of 10 mM, and routi-
nely stored at 4°C without freezing. DRAQ5 is a dark blue crystalline solid of molecular weight 412.54 and is stable at room temperature. DRAQ5 was used at a concentration of 20 µM for all experiments, with a 10-min loading time. Hoechst dye No. 33342 (Sigma-Aldrich) was prepared as an aqueous stock solution and added directly with mixing to culture media containing cells (5 µM × 1 h at 37°C). A stock buffer solution of pH 7.2 was prepared containing 0.01 M Trizma base (Sigma-Aldrich), 0.1 M NaCl (Sigma-Aldrich), and 0.001 M EDTA (Sigma-Aldrich). A stock solution of calf thymus DNA (CTDNA) (Sigma-Aldrich) was prepared in the buffer of 1 mM concentration. The DNA solution was gently mixed at 4°C overnight and then sonicated on ice in 10-s bursts for 5–6 bursts at 18 Hz (to reduce the DNA chain length). The stock was then gently rotated for 1 h and stored at 4°C.

Spectral Analysis of DRAQ5—Solution Studies

Absorption spectra for 0.2 ml samples in 1-cm path length micro quartz silica cuvettes were determined using a Beckman Coulter DU 800 UV/Visible spectrophotometer. Wavelength scans were collected from 200 to 800 nm.

Emission spectra for 0.5 ml solution of probe in 1-cm path length semimicro quartz silica cuvette were determined using a Perkin-Elmer LS50B spectrofluorometer, with a spectral bandwidth of 5 nm. The spectrofluorometer was equipped with an extended red photomultiplier tube (PMT; type R928; Hamamatsu Photonics KK, Japan; quantum efficiency of 5.5% at 700 nm, manufacturers’ information). Samples were excited at 647 nm wavelength and emission-scanned as indicated.

Both DRAQ5 and DNA-fluorochrome fluorescence was measured by the addition of concentrated CTDNA solutions to the probe, in a molar ratio of probe/DNA of either 1:54 or 0:154, with mixing. The spectra shown for both absorption and emission were corrected for the buffer background and, additionally for the emission, for the spectral sensitivity of the PMT. Quantum yield measurements were determined for DRAQ5 at 637 nm excitation wavelength; Nile Blue (Φ, 0.27°) in methanol (Invitrogen, Paisley, UK) was used as a reference standard. We note that the detection system has a fall of 45% at higher wavelengths, when comparing peak emissions for Nile Blue (665 nm) and DRAQ5 (695 nm) according to manufacturer’s technical data (Hamamatsu Photonics KK, Japan).

Live Cell Spectral Imaging of DRAQ5

In vivo spectral imaging of DRAQ5 loaded U-2 OS GFP-cyclin B1 cells was carried out using a Leica TCS SP2 RS confocal spectral imaging system (Leica Microsystems (UK), Milton Keynes, UK). Calibration of the spectral imaging system was carried out using reflection images of chromium-coated coverslips, excited using both laser lines (635 and 488 nm). DRAQ5 was excited by a 635 nm HeNe laser. Scans of wavelength versus intensity (λ scans) were acquired with 50 sequential wavelength data point acquisitions, with wavelength sampling increment (spectral width) of 5 nm, from 650 to 750 nm. These parameters were used in order to achieve Nyquist sampling and to achieve greater spectral resolution. GFP was excited by a 488-nm laser line and emission was collected from 500 to 590 nm, with 30 sequential wavelength data point acquisitions (spectral width of 5 nm). These images were used to identify cell cycle position of the cells, as related to the intensity and location of the GFP-cyclin B1 reporter in the cells. All spectra were collected with the pinhole fully open, to increase the potential number of photons detected, and hence increase signal-to-noise. Line scanning average of 4 was used, and images were of 8-bit format.

Molecular Modeling of DRAQ5

The geometries of three different conformations of DRAQ5 were optimized by 6-31G(d) methods, as implemented in GAMESS-US software package (18). Final optimized geometries were docked into the DNA structures extracted from the PDB files of the DNA complex with Hoechst 33342 (PDB access codes 127D, 129D, 303D). The docking was performed using global range molecular matching (GRAMM) software (19, 20) and high resolution rigid body searching for favorable binding configurations between a small ligand and a DNA, without any constraints or limitations. The resulting DNA–ligand complexes were subjected to Amber all-atom energy minimization, as implemented in Macromodel 8.5 (21), until the rms of conjugate gradient was 0.1 kcal/mol/A. One of the DNA-DRAQ5 complexes was also subjected to the molecular dynamics simulations at 100 K for 100 ps, followed by energy minimization.

Flow Cytometry—Hoechst 33342 Quenching

DRAQ5 can be excited at 488, 514, 568, 633, or 647 nm (with efficiency increasing with the wavelength of the excitation). A FACSVantage flow cytometer was used (Becton Dickinson, Cowley, UK), which was equipped with a Coherent Enterprise II argon ion laser having 488 nm and multiline UV (350–360 nm) outputs (Coherent, Santa Clara, CA). CELLQuest software (Becton Dickinson Immunocytometry Systems) and filters (Omega Filters, Brattleboro, VT) was used for signal acquisition and analysis of Hoechst 33342 quench, and DRAQ5 loading in HL60 cells. The optics for the analysis of DRAQ5 excited by the primary 488-nm beam was composed of reflection at a short pass 610-nm (cat. no. 02-60947-21) dichroic and a long pass 715-nm barrier filter. The optics for the analysis of Hoechst 33342 excited by a delayed UV beam was composed of reflection at a long pass 510-nm dichroic and a barrier bandpass filter of 424/44 nm. Forward- and side-scattered light was collected for 10,000 cells and were analyzed to exclude any cell debris. All pulse height parameters were analyzed using CellQuest software (Becton Dickinson). Data are expressed as mean FI values.
Fluorescence Imaging—Hoechst 33342 Quenching

*In vivo* fluorescence imaging of U-2 OS GFP–cyclin B1 cells loaded with Hoechst 33342 and DRAQ5 was carried out on an axiovert S100TV inverted microscope system (Zeiss C, Welwyn Garden City, UK) equipped with a Hamamatsu IEEE1394-based digital camera ORCA-ER (Hamamatsu Welwyn Garden City, UK) and using a Fluar 40×/1.30 Ph3 oil objective. Cells were loaded with Hoechst 33342 alone for 15 min, washed with fresh media, and then imaged using a 460–500 nm UV filter, prior to the loading of 20 lM DRAQ5. Images were then taken of the Hoechst 33342 and the DRAQ5 alone and used as controls. The image analysis package MetaMorph (Molecular Devices Corporation, PA, USA) was used to quantify both fluorophores for mean nuclear FI, corrected for background.

Cell Cycle Profiling

U-2 OS cell monolayers were detached using trypsin and cells resuspended in complete medium supplemented with 10 mM Hepes (pH 7.2) prior to DRAQ5 loading (20 μM X 15 min at 37°C). For cell cycle profiling, a MoFlo cytometer (DakoCytomation, Fort Collins, CO, USA) equipped with an argon ion laser (Spectra-Physics Lasers, Newbury, UK), using 488 nm excitation, was used. The argon laser power was regulated at 600 mW. Summit 4.0 software (DakoCytomation) was used for signal acquisition and analysis. Signal was collected through emission filters: (i) 620LP; (ii) 670/30BP (cat. no. SP96593); (iii) 695LP; (iv) 700LP (cat. no. SP96608); (v) 715LP—all collected after reflection via a 605DSP dichroic (cat. no. SP96625). Signals for forward, side scatter, and fluorescence were collected for 20,000 cells, using forward light scatter parameter as the threshold signal. Data were analyzed using WinMDI (http://facs.scripps.edu/software).
html; authored by Dr. J. Trotter, The Scripps Research Institute, California, USA), with FSC/SSC and pulse width gating to exclude doublets. Cell cycle analysis was carried out using Cylchred (www.cardiff.ac.uk/medicine/hematology/cytonetuk/documents/software.htm; authored by Dr. T. Hoy, Department of Haematology, Cardiff University, School of Medicine, Cardiff, UK) to give the proportion of cells in each phase of the cell cycle. Flow data was then exported into spreadsheets for further analysis of GFP and DRAQ5 DNA content data.

RESULTS

DRAQ5 Spectral Characteristics and Quantum Yield Measurements

Figure 1 shows the UV–visible absorbance spectrum and emission spectrum for DRAQ5 in Tris buffer at pH 7.2, with and without CTDNA. The absorbance spectrum of DRAQ5 alone (Fig. 1a) gave maxima at 594 and 638 nm. In addition, other maxima occurred at 232 and 304 nm (not shown). On addition of CTDNA in a ratio of 1.54 (DRAQ5/DNA), the absorption spectrum was observed to shift slightly to longer wavelengths, with the maxima moving to 596 and 642 nm (Fig. 1b). The addition of CTDNA in a higher ratio (0.154) causes the absorption spectrum to shift more dramatically toward longer wavelengths, with the maxima now being located at 610 and 656 nm (Fig. 1c). The addition of CTDNA in a higher ratio (0.154) causes the absorption spectrum to shift more dramatically toward longer wavelengths, with the maxima now being located at 610 and 656 nm (Fig. 1c). With the addition of CTDNA, it is not only the location of the maxima that changes but also their relative heights. The height of the second maxima consistently increased with the addition of CTDNA, until this became dominant. The emission spectrum of DRAQ5 also demonstrated shifts in wavelength with the addition of CTDNA (Figs. 1e and 1f). Additionally, the appearance of a shoulder at 750 nm was observed when CTDNA, in a ratio of 1,54, caused the absorption spectrum to shift more dramatically toward longer wavelengths, with the maxima now being located at 610 and 656 nm (Fig. 1c).

The quantum yield gives the probability of the excited state being deactivated by fluorescence in preference to other, nonradiative mechanisms. The quantum yield values for DRAQ5 in Tris buffer alone, and in the presence of CTDNA, were all similar, ranging from 0.003 to 0.004, demonstrating that most of the absorbed photon energy translates to nonradiative decay. An unchanging quantum yield shows that DRAQ5 fluorescence is not significantly enhanced upon binding to CTDNA.

Live Cell Spectral Analysis of DRAQ5 and the Impact of Cell Cycle Stage

Spectral confocal laser scanning microscopy (spectral CLSM) was used to determine the emission spectrum of DRAQ5 bound to interphase chromatin DNA of human osteosarcoma U-2 OS cells. These cells expressed GFP–cyclin B1, providing a second and independent fluorescent parameter for determining cell cycle position (16), allowing us to further explore the dynamic range of DRAQ5 as a DNA-targeted fluorescent probe. A simple calibration protocol was implemented, suitable for the basic analysis undertaken. The pinhole aperture was opened in this protocol because we needed to maximize signal to noise by collecting thicker optical sections. As previous comprehensive studies have shown, the pinhole size clearly affects the spectral performance; therefore, all experiments were conducted in the exact same conditions (22,23). Our reflection/mirror standard of a chromium-coated coverslip allowed us to operate the CLSM intact using the illumination sources required for the fluorescence excitation of our fluorophores. The CLSM instrument was calibrated using reflection imaging, to determine the spectral channel width (Fig. 2). Full width half maximum (FWHM) measurements of the resultant profiles were shown to have 8 nm width around a peak of 633 nm in the near-red spectrum, which improved to 5 nm at 488 nm, ensuring that spectral positioning across the 488–633 nm range of the detector is correct. λ stacks were acquired for both the GFP and DRAQ5 signal (Fig. 3). GFP expression identified G1 and S-phase cells (low GFP expression) (Figs. 3a–3c) and late cell cycle, G2 cells (high GFP expression prior to nuclear translocation at mitosis) (Fig. 3d), but gave identical spectral profiles in which the peak amplitude increased. The corresponding DRAQ5 emission did not change in these interphase cells, showing that cell cycle...
position does not affect the spectra of this probe. The spectral profile of DRAQ5 bound to chromatin corresponded and overlapped with the peak emission obtained using fluorimetry. However, a unique shape consisting of two peaks located at 681 and 707 nm was observed (Fig. 3). The identification of the two peaks is not a surprise, since this emission profile now mirrors the absorption spectrum of DRAQ5. We hypothesized that the peaks represented unbound and bound forms, which would therefore not fully colocalize, since bound probe signals would be concentrated in scanned volumes occupied by high chromatin densities. Images representing the first and second peaks were produced from the original image stacks (Figs. 3e and 3f). A ratio image was made of the peak 1 (far red) image over the peak 2 (near IR) image (Fig. 3g). This resulted in an almost binary image, suggesting that every pixel present in the images consists of both components (peak 1 and 2) of the DRAQ5 spectra. Thus, we suggest that if the lower peak component represents elements of unbound probe, then it is most likely that this is in rapid equilib-
rium with a bound form, which also demonstrates similar emission characteristics. The near IR peak may therefore represent a modified binding mode. Fluorescence lifetime measurements may shed light on this issue.

**DRAQ5–DNA Binding Mode**

Molecular docking studies have shown that aromatic moiety of the DRAQ5 molecule preferentially binds to the AATT part of the DNA sequence, where there is potential overlap with Hoechst dye binding (Fig. 4). Molecular dynamics simulation of the DNA-DRAQ5 complex, without any constraints, leads to DRAQ5 protrusion into the interface of two AT base pairs (Figs. 4a and 4b) by breaking the two hydrogen bonds between one AT base pair. The aromatic rings of those two base pairs are displaced out of the DNA backbone, with DRAQ5 stacking between aromatic rings of those two bases.

To determine the efficiency with which DRAQ5 could act as a potential quenching agent on cellular DNA, we initially screened the ability of DRAQ5 to modify the enhanced fluorescence of Hoechst 33342 bound to nuclear DNA of intact cells using flow cytometry (Fig. 4c). We exploited the ability of DRAQ5 to be excited at suboptimal wavelengths (e.g. 488 nm) so that excitation conditions could be established for the different imaging and flow cytometry platforms used, and to permit coexcitation protocols in later GFP studies. Thus, the appearance of DRAQ5 within intact cells was determined using 488-nm excitation and UV-activation of Hoechst 33342-DNA (analyzing the violet/blue-biased fluorescence emissions). DRAQ5 uptake was rapid and reached maximal loading within 5-10 min. The quench pattern mirrored the appearance of anthraquinone-associated fluorescence. The removal of Hoechst 33342 from the medium prior to DRAQ5 addition did not affect the quench or anthraquinone uptake patterns observed, suggesting that DRAQ5 equilibrium across the plasma membrane was not affected by the presence of free Hoechst 33342 molecules (data not shown). Wide-field imaging revealed that the quenching process was dramatic in the nuclear compartment. The Hoechst dye labeling demonstrated a punctate pattern (Fig. 5a) (mean Fl of $315 \pm 80 \pm SD$); however, after a 10-min exposure to DRAQ5, these structures disappeared with a comprehensively attenuated fluorescent signal in the nucleus (Fig. 5c) (mean Fl, $16 \pm 9 \pm SD$). Reversing the order of addition by the exposure of DRAQ5-pretreated cells to Hoechst 33342 showed no apparent displacement of the far-red ligand from the nuclei of live cells (data not shown).

**Spectral Windows for the Robust Reporting of Cell Cycle Position**

Combining GFP reporting technology with simultaneous live cell DNA content analysis or nuclear discrimination can provide a flexible, extended map for cell cycle characterization. The characteristics of GFP demand the application of a far-red fluorescent cell-permeable DNA/nuclear reporter to eliminate spectral overlap. The ability of the GFP probe to report cell cycle position with "clean" switch-on and switch-off characteristics between G1 and G2 was clearly demonstrated by the expected plume, extending from G1 to G2 (Fig. 2c).

Here we have explored the spectral analysis constraints for DRAQ5-DNA, reporting for the allocation of cells to functional cell cycle compartments. Thus, we applied a cell cycle phase fitting algorithm (Cylchred software) to the DRAQ5 signal (pulse peak) frequency distributions obtained for different spectral regions, using flow cytometry. Coefficients of variation obtained for the algorithm extracted G1 peaks were $8.1, 8.8, 8.8, 8.3, \text{ and } 9.2\%$, respectively, for the filters 620LP, 670/30BP, 695LP, 700LP, and 715LP. The results suggest that the 700LP is a compromise.

**Fig. 4.** Binding modes of DRAQ5 and Hoechst 33342 using molecular modeling and flow cytometric study of Hoechst quenching by DRAQ5. The superposition of DRAQ5 (green) and Hoechst 33342 (orange) (a), and DRAQ5 alone (b) into the minor groove of the DNA. The position of the Hoechst 33342 was determined by X-ray crystallography (PDB access number 127D) and the position of DRAQ5 was found by rigid body docking. Tracking Hoechst 33342 and DRAQ5 fluorescence signal using flow cytometry to determine the kinetics of Hoechst signal quenching after the addition of DRAQ5 in HL60 cells Panel c shows a flow cytometric quench study of Hoechst 33342 fluorescence in preloaded cells (triangles), preloaded cells subsequently exposed to DRAQ5 (squares) when compared with the parallel monitoring of DRAQ5 uptake (diamonds).
between highest spectral cut-off in the far red, lowest CV and maximum photon capture to facilitate high signal-to-noise ratio. CV values will be compromised by the inherent plasticity in the DNA content of the established tumor cell line, and the relatively high speed of event acquisition typically selected for the MoFlo™-based cytometry system. We employed a parallel biological control, previously reported (5,6), of B-cell lymphoma cells stained directly in suspension. These controls showed that optimal G1 and G2 CV values of 5.2–5.4 were also obtained for the 700LP filter, set up with a corresponding maximal G2/G1 ratio of 1.75 for mean peak FI measurements (data not shown).

Cells were also allocated to cell cycle compartments on the basis of the proportion of the population abstracted by Cylchred software analysis and the ranked DRAQ5 signal. The 488-nm coexcitation of GFP gave the corresponding maximal G2/G1 ratio of 1.75 for mean peak FI measurements (data not shown).

Cells were also allocated to cell cycle compartments on the basis of the proportion of the population abstracted by Cylchred software analysis and the ranked DRAQ5 signal. The 488-nm coexcitation of GFP gave the corresponding cyclin B1 reporter fluorescence measurements for these DRAQ5 allocated bins. The G1, S phase, and G2 subpopulations demonstrated geometric mean fluorescence values of 110 ± 15.7, 323 ± 12.3, and 549 ± 13.8, respectively, showing a fivefold change from basal to peak expression. This high dynamic range provides for a sensitive test for event allocation. The S phase was further divided into three equal compartments, comprising subjective descriptions for early (SE), mid (SM), and late (SL) S phase. Figure 6 shows the impact of DRAQ5 emission window selection on the measurement of the cell cycle tracking GFP signal for each cell cycle compartment. The results indicate similar profiles for all spectral windows studied, with the lowest G1 basal signal being allocated by DRAQ5 analysis using 700LP.

**DISCUSSION**

The anticancer agent mitoxantrone and modified anthraquinones show variable DNA-affinic and cell-permeation properties (5,24–27), with the probe DRAQ5 readily crossing the plasma membrane of intact living cells (5,6). The current study on this 1,5-bisalkylaminoanthraquinone has addressed target binding and spectral properties in live cells. Molecular modeling suggests that DRAQ5 is capable of binding to DNA through intercalation, as expected for this class of anthraquinone molecules. The intercalation is stabilized by electrostatic interactions between the protonated tertiary amino group of the side chain and the phosphate backbone of the DNA. Previous molecular modeling studies have suggested that anthraquinone derivatives substituted at the 1,4- and 1,8-α-NH(CH2)2NH(CH2CH3)2 side chain intercalate with DNA, with both substituents in the same groove (classical intercalation), while the similarly substituted 1,5-derivative intercalates, in a threading mode,
with a side chain in each groove. The current study shows that binding appears to involve a preference for AT-containing sequences in living cells suggested by the results of Hoechst 33342 quenching studies, in which DRAQ5 appears to have the ability to colocalize at a significant fraction of Hoechst 33342 binding sites, and to have the potential for minor groove interactions revealed by modeling studies. The AT-targeting, within seconds of dye exposure, would be expected to impact upon protein–DNA associations within such regions. Indeed, we have previously reported a rapid rearrangement of an intranuclear location protein MSH-2 from AT-rich chromatin sites, as defined by a colocalization study (28), a finding consistent with AT-selectivity. In this previous study, we did not observe DRAQ5 quenching of the Hoechst 33342 fluorescence as, critically, the timing of the DRAQ5 exposure was different. The previous study reported on cells being briefly exposed to Hoechst 33342 (8 μM for 10 min) to access high affinity sites without any extensive spectral shift, and then Draq5 was added (at 20 μM for 5 min) to reduce access to the initial interaction at these high affinity sites. In the current article, the conditions of Hoechst 33342 used (5 μM for 1 h) mimic a typical full labeling protocol to reach equilibrium for live cells. The comparison of DRAQ5 AT-specific fluorescence banding in human and/or mouse metaphase chromosome spreads with classic DAPI banding would enable the evaluation of the biological usefulness of the AT-specificity (29).

The relatively structureless and broad absorption and emission spectra observed for DRAQ5 are typical for molecules in solvents, while our findings are consistent with the expectation of a structured emission spectra upon ligand docking on DNA. Emission spectra are typically mirror images of the absorption spectra, but shifted to higher wavelengths (Stokes' shift) (12). This similarity in spectral shape occurs because the same electronic transition is involved in both processes of absorption and emission. The change in the absorbance spectrum observed in this study, of the 1,4-dihydroxyanthraquinone, reflected as a shift by 16 nm to the red on binding to DNA, may be due to this substituted anthraquinone undergoing extended conjugation with the purine DNA bases of the intercalation site. A shift in emission spectra also occurs that the relative Stokes' shift remains equivalent. Here we demonstrate that DRAQ5 does not undergo fluorescence enhancement as reflected by unchanged quantum yield values, unlike some other DNA binding probes, such as Hoechst 33342, which demonstrates an increase in quantum yield by up to 10-fold (30). The low quantum yield values are a disadvantage when considering the chromophore aspect of the agent; however, this is mitigated by the high binding coefficient, as the DRAQ5 molecule has a high affinity/AT preference for DNA (binding constant of $K = 3.0 \times 10^7 \pm 2.4 \times 10^6$ for polydA-polydT (28)). Here we show the massive separation of the DRAQ5 and GFP signals in live cells, indicating that there is no contribution from unbound dye molecules to the signals collected in a GFP spectral window. Thus high quantum yield is not always a favorable property of fluoros in multiplexed assays.

The concept of targeting DNA damaging agents to specific DNA sequences has led to a search for enhanced DNA-affinic properties of DNA intercalators and minor groove binders (1,31–33). Reporter molecules with distinct and informative fluorescence profiles that can rapidly form equilibria within nuclear compartments can aid the building of pharmacokinetic models to support drug development and deployment. We have shown that a molecular modeling approach can be used to suggest that DRAQ5 is a suitable minor groove quenching agent, with spectral properties distinct from GFP. Previous work has also shown that DRAQ5 has a low two-photon cross-section and is therefore a two-photon dark probe at wavelengths below 920 nm, but acts as a quenching agent to reveal the complex DNA binding pattern and distribution of the anticancer agent, topotecan (34). Others have employed DRAQ5 to colocalize with SYTO13 in the nucleus, with DRAQ5 quenching the SYTO13 signal, and this effect being attributed to Förster resonance energy transfer from SYTO13 to DRAQ5 (35).

The spatial and temporal control of a cell cycle protein, cyclin B1, has been previously exploited to develop a highly specific and sensitive fluorescent reporter of cell cycle traverse in living cells (16). It comprises a GFP linked to critical sequence regions, imposing the expression, localization, and degradation properties of cyclin B1, such that the construct shadows the kinetics of the endogenous cyclin through the late cell cycle. In the present study, a U2 OS cell line stably transfected with this reporter (GFP-cyclin B1) provided an independent marker for cell cycle age, enabling the rigorous interrogation of DRAQ5 as a quantitative DNA-affinic agent, while considering particular spectral windows of the emission profile. In practical terms, we conclude here that DRAQ5 DNA content analysis at a spectral window of >700 nm provides for a robust tool for sectoring the cell cycle, with maximal spectral separation for visible range fluoros including GFP. Near-IR wavelength selection should also

![Graph](image-url)
accommodate new fluorescence tagging technologies for intracellular proteins that start to occupy longer red wavelength windows. On the other hand, we suggest that sampling the entire DRAQ5 spectrum for nuclear location tracking, even under conditions of equilibrium with free dye molecules (e.g. in "no wash" protocols), would provide good signal-to-noise in HCS screens/translocation assays, especially those employing cytoplasmic reporters. Spectral profiles of molecular probe behavior, such as those described here for DRAQ5, highlight the potential for noninvasive cytomteric tools for tracking molecular interactions in live cells.

LITERATURE CITED