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Nuclear Cytometry and Chromatin Organisation

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Running headline: Chromatin & Probes

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Key Words

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Electron Microscopy

Super Resolution Microscopy

Abbreviations

SAF-A: scaffold attachment factor A

HMM: Hidden Markov Model

FLIM: Fluorescence Lifetime Imaging Microscopy SIM: Saturated Structured Illumination Microscopy

STED: Stimulated Emission Depletion

STORM: Stochastic Optical Reconstruction Microscopy

PALM: Photoactivation Localization Microscopy

fPALM fluorescence-based PALM

SMLM: Single Molecule Localization Microscopy

SPDM: Spectral Precision Distance/Position Determination Microscopy

BALM: Binding-Activated Localization Microscopy

DAB: diaminobenzidine

ROS: Reactive Oxygen Species

DAPI: (4',6-Diamidino-2- phenylindole dihydrochloride)

IdU: 5-iodo-2-deoxyuridine

AO: Acridine Orange

CARS: Coherent anti-Stokes Raman scattering

MSI: Mass Spectrometry Imaging

PWS: Partial-wave spectroscopic

MTG: MitoTracker Green

ERTG: Endoplasmic Reticulum Tracker Green

NETs: Neutrophil Extracellular Traps

m-AMSA: 4'-(9-acridinylamino) methane sulfon-m-anisidide

MTX: mitoxantrone

μ PK-PD-CD: Micro-PharmacoKinetics and cellular PharmacoDynamics with specific Cellular Descriptors

Abstract:

The nuclear-targeting chemical probe, for the detection and quantification of DNA within cells, has been a mainstay of cytometry - from the colorimetric Feulgen stain to smart fluorescent agents with tuned functionality. The level of nuclear structure and function at which the probe aims to readout, or indeed at which a DNA-targeted drug acts, is shadowed by a wide range of detection modalities and analytical methods. These methods are invariably limited in terms of the resolution attainable

versus the volume occupied by targeted chromatin structures. The scalar challenge arises from the need to understand the extent and different levels of compaction of genomic DNA and how such structures can be re-modelled, reported or even perturbed by both probes and drugs. New cytometric approaches to analysing chromatin released from cells, as in NETosis, demonstrate the potential for probes to report defining features. Typical of recent insights into chromatin organisation is the 'ChromEMT' study that exploits the properties of the anthraquinone-based cytometric dye DRAQ5™. Insights reveals that local and global 3D chromatin structures in the nucleus determine compaction. Cytometry can report on complex levels of chromatin order, disorder, disassembly and active disruption. The focus of this review is nuclear cytometry, with linked reference to DNA targeting drugs and probes, their impact in the chromatin environment.

Introduction

'form ever follows function'

Louis Henry Sullivan (1856 – 1924)

American architect and 'father of skyscrapers'

Cellular structures, first recognised by von Nägeli in 1842, would later be called 'chromosomes' by Waldeyer in 1888 to reflect their staining behaviour with dyes. The observable packaging of a cell's genetic material and its metrology have provided a continuing source of interest through to modern cytogenetics. Cytometry in its widest definition has a considerable track record in the analysis of the multi-level organisation of the genetic material in eukaryotic nuclei (1-8). Nuclear cytometry-based methods can simplify the analysis and quantification of protein associations to chromatin and reveal population heterogeneity (9). Recent methods describe the extraction of nuclei for the purpose of probing cellular and transcriptional states (10,11) although preserving native chromatin superstructure remains a challenge. Extensive information is available from academic and commercial sources on the spectral properties of molecular probes for nuclear cytometry that can report nuclear states. This review will not explore these in detail. However, understanding the balance of advantages and limitations is important when a given probe is used for specific purposes or in a sensitive cellular system (12,13). Critical factors in live cell studies are the biological impact of the probe and its access to a nuclear target determined by chromatin organisation (14). Such factors are shared by nuclear-targeted drugs in driving their pharmacodynamic effects. Here these aspects are discussed with respect to DNA-interactive probes, with linked reference to DNA targeting drugs, in the context of the chromatin environment.

Chromatin organisation

Genomic DNA is compacted into chromatin through packaging with histone and non-histone proteins. Chromatin folding and packaging has to change dynamically as the cell progresses through the cell cycle. Chromatin organisation is addressable by DNA-affinic probes. The challenge is how to recognise and measure different scalar levels of chromatin organisation, from base-pair to the whole nucleus. The scalar levels of chromatin organisation dictate the analytical approach employed and the extent to which a probe can be usefully deployed (Fig. 1). The cell deals with an impressive length of linear DNA packaged within its nuclear volume. Towards one end of the scale, haploid yeast cells with a mean nuclear volume of $3 \mu\text{m}^3$ has a genome length of $\sim 12 \text{ Mbp}$. This represents some 0.3 % of the nuclear volume or around $0.01 \mu\text{m}^3$. A human diploid cell nucleus with a volume of around $700 \mu\text{m}^3$ copes with 3 billion bases and a combined strand length of genomic DNA of just over 2 m.

There is increasing evidence of a relationship between higher orders of chromatin topology and the regulation of global patterns of gene expression (15). One concept is that evolutionary selection favours the clustering of widely expressed housekeeping genes. Such clusters adopt an open configuration of chromatin structure. Open chromatin fibres have also been found to be enriched in

gene-rich domains and not just in those regions that are transcriptionally active (16). The mechanisms by which large-scale chromatin structures can be de-compacted or undergo dynamic folding are becoming clearer (17). An informative example is how the scaffold attachment factor A (SAF-A) can interact with chromatin-associated RNAs in a transcription-dependent manner. SAF-A oligomerization de-compacts large-scale chromatin structure while SAF-A loss or monomerization promotes aberrant chromosome folding and the accumulation of genome damage (17). This also highlights a crucial role for chromatin organisation in maintaining a stable environment for the genome.

Annotation of the human genome with respect to different chromatin states can reveal signatures for functional descriptions. Descriptors can reflect transcription-associated or active large-scale repressed states (18). Recently the ChromHMM tool has been described that can seek signatures of chromatin-states using a multivariate hidden Markov model (HMM) (19). The tool performs an enrichment analysis of the resulting annotations to allow functional interpretation (19). Advances in the high-resolution approaches of electron and super-resolution fluorescence microscopy, together with DNA sequencing, have provide views of the relationship between chromatin and nuclear machineries within a '4D nucleome' (20). This approach has provided a finer structural definition of active and inactive nuclear compartments. An inactive compartment comprises the core of chromatin domain clusters. The active compartment locates at the periphery of these clusters. A further compartment links to routes for nuclear import and export via nuclear pores (20).

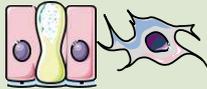
Analysis level	DNA analysis scale level	Detection ranges for exemplar methods	Molecular probe & reporter deployment
	micro-community to single cell [2000-20 μm]	<i>tissue histopathology, flow/image/mass cytometry; Raman scattering; incorporation methods; photoacoustics</i>	Cell recognition, location, tracking & status, differentiation, viability & function
	nucleus & cell cycle dynamics [20-1 μm]	<i>live and fixed cell fluorescent reporters,</i>	Nuclear structure and state changes, functional distributions, kinetics, targeting
	sub-chromosomal regions [2-0.1 μm]	<i>Super Resolution Optical Microscopy Opto-methods (eg FRET) ChromEMT</i>	Banding, telomere, centromere structural changes; high resolution signal location & relationships
	chromatin fibre & packing [30-10 nm]	<i>atomic force microscopy imaging electron microscopy phase plate cryo-EM imaging electron spectroscopic imaging</i>	Chromatin compaction; dynamic changes and perturbation
	nucleosome & protein binding [6 nm]	<i>Structural approaches: nuclear magnetic resonance spectroscopy,</i>	DNA metabolism, winding and unwinding dynamics; receptor binding
	duplex DNA & sequence/groove interactions [10bp: 2.4 x 3.4 nm]	<i>X-ray crystallography</i>	Sequence selectivity and structural modification, damage & repair; ligand binding

Figure 1: Probing the hierarchy of chromatin organization

At its lowest level, DNA wraps around histone octamers to form nucleosomes but needs to eventually compact into discrete chromosomes – with conceptual models for the elaboration of chromatin fibre structure supported by *in vitro* studies (Fig. 1). Chromatin experiences a hierarchy of packaging but the models become increasingly speculative as the volume of observation increases. Chromatin carries negative charges due to incomplete neutralisation of the DNA polymer's phosphate backbone by basic core histones. The result is that chromatin structure is subject to electrostatic repulsion between its neighbouring sections. Chromatin also responds to changes in the electrostatic microenvironment which interacts both with negatively charged DNA and the positively charged histones. As a result chromatin structure can be manipulated *in vitro* by simply changing the electrostatic environment, frequently by supplying divalent cations. In live cells these fundamental properties contribute to a state of fluidity – perceived as a ‘liquid-like behaviour’ of the 10-nm chromatin fibre (21). The 30-nm chromatin fibre is often regarded as the secondary structure of chromatin directed by nucleosomes, nucleosome-protein complexes and regulatory factors (22). This key structure remains controversial both in its form and the extent to which it is adopted in different cells types (23,24). However, even at these primary and secondary levels, it is recognised that the

chromatin structural environment does not remain static. In live cells chromatin transitions can occur on timescales which range from milliseconds to minutes or even hours (25).

For a DNA probe to work it must reach its target. Inadequate target exposure can also be a factor in the pharmacodynamic failure of a drug. Predicting access to intracellular targets can involve a label-free (26) or fluorescent drug-based modelling approaches (27). However, such methods are yet to consider the impact of the chromatin 'labyrinth' (26). Chromatin structures present an 'obstacle network' for diffusing proteins, effective over a distance of 1–2 μm , providing a temporal restraint on interactions with DNA (20). It remains unclear whether 'obstructed transport' through chromatin networks (28) can predict probe/drug access to DNA targets. The caveat is that a probe may be limited by the chromatin states it can report – a source of unsuspected bias. The stress responses of chromatin control chromatin accessibility to facilitate genome stability (29). For example, the 'relaxation' of chromatin at regions of damaged DNA signals damage location and promotes the assembly of repair complexes (30). Nucleosomal structure itself can be considered both a 'central signalling hub' and a 'landing platform' in the repair process (31). A range of fluorescence imaging methods can now provide descriptions of DNA repair in single cells [review: (32)]. Fluorescent probes for marking sites of DNA damage can provide insights into defects in DNA repair. Such approaches include detection of base excision repair intermediates (apurinic/apyrimidinic sites) in DNA (33) and dual-incision steps of UV-damaged cellular nucleotides (34) in combination with flow cytometry.

Chromatin dynamics

Chromatin remodelling encompasses multiple activities including: DNA replication and repair, transcriptional control, epigenetic regulation, programmed disassembly during apoptosis, chromosome packaging and segregation. These events are clearly relevant to critical cellular transitions in development, neoplastic progression and pluripotency (20,35-37). A current view is that regulation of the chromatin nano-environment, over ranges that exhibit chromatin packing-density heterogeneity, can allow for a predictable modulation of global patterns in gene expression (38). A 'rational' modulation of chromatin density fluctuations can lead to a decrease in global transcriptional activity. One can view this as 'macrogenomic engineering' within the nucleus for directing whole-scale transcription levels (38).

ATP-dependent nucleosome-remodelling complexes direct histone behaviour through an ATPase-translocase 'motor' function that mobilises DNA within the nucleosome (39). ATP-dependent enzymes that remodel chromatin are therefore important controllers of structure (36,40,41). Key functional components of chromatin, along with histones, condensins, cohesins and regulatory proteins (42-44), are the distinct class of topology correcting enzymes – the DNA topoisomerases. Chromatin is a store of torsional energy which results in the release of negative superhelicity upon decompaction (45). Conversely, dynamic changes in DNA supercoiling will dictate packaging and transcription (46). Nuclear problems can arise when the replication of chromatin loops generates interlinked DNA products (catenanes) or when DNA function requires the resolution of torsional stress. The cell's enzymatic solution is via the DNA topoisomerases. These enzymes provide a co-ordinated process of

strand passing within a DNA-enzyme complex (type IA and type IB enzymes). They can also allow the movement of an actively-cleaved strand around an intact strand prior to re-ligation (type IB enzymes). Type IIA enzymes are 'full decatenases' (40), passing one duplex through a double-strand break generated by the enzyme in another duplex. On the other hand, type IA enzymes strand pass single-stranded DNA segments by the adjacent duplex and thereby locally changing the linking number (40). Type IIA DNA topoisomerases are vital for progression through the cell cycle (47). In terms of probe manipulation of superhelicity, intercalation can generate changes in local supercoil relaxation states with extension of the duplex. A cell permeant biotinylated 4,5,8-trimethylpsoralen probe, that preferentially intercalates with DNA enriched in negative supercoils, has been used to monitor changes in DNA supercoiling in vivo (45). RNA polymerase and topoisomerase activities remodel DNA supercoiling, creating supercoiling domains that affect the folding of large-scale chromatin structures (45).

The DNA topoisomerases present specific and demonstrable targets for several classes of both anticancer and antibacterial drugs (41). A flow cytometry-based method has been described for the high-throughput analysis of drug-stabilized topoisomerase II cleavage complexes in human cells (48). In mammalian cells the DNA damage signalling responses to DNA topoisomerase inhibition can also be tracked by flow cytometry to reveal the restraints on DNA replication and cell cycle progression (49-51). The human type IIA enzyme is a target for catalytic inhibitors such as the bis-dioxopiperazine ICRF-193. ICRF-193 induces late cell cycle checkpoint stalling, decatenation inhibition, mitotic anomalies or even bypass of mitosis to polyploid states in permissive cells (52). Recent structural analyses indicate how type IIA enzymes embrace the helix DNA and how the enzyme-DNA interactions inform drug behaviour (53). The human type IIA topoisomerase proteins, topoisomerase II α (Top2alpha) and topoisomerase II β (Top2beta), are the targets for several anticancer agents including etoposide, the DNA intercalating anthracyclines (doxorubicin, daunorubicin) and the anthraquinone mitoxantrone (54). The cellular roles of topoisomerase II β (55) and the consequences of inhibition, given its expression in terminal differentiation, are less understood compared with the cell cycle regulated topoisomerase II α . Bacterial type II topoisomerases (gyrase and topoisomerase IV) are the targets of quinolones and aminocoumarin antibiotics (41).

The eukaryotic type IB topoisomerases (Top1) are classically targeted by camptothecin and related derivatives such as topotecan or irinotecan. Genome-wide high-resolution mapping has revealed the targeting of transcriptionally active genomic regions by the Top1 inhibitor topotecan and the Top2 inhibitor etoposide. On the hand, daunorubicin induces DNA breaks and evicts histones from active chromatin with a 'quenching' of local DNA damage responses (56). Fluorescence lifetime imaging microscopy (FLIM) has been used to map the nuclear docking of topotecan at a subset of DNA sites in nuclear structures of live breast tumour cells in which the DNA binding probe DRAQ5 has been used to uncover sites of drug interaction (57). The anticancer anthracyclines daunorubicin (daunomycin), doxorubicin (adriamycin) and epirubicin (4'-epi-doxorubicin, an active isomer of doxorubicin), belong to a class recognized as potent Top2 'poisons'. Their ability to generate protein-associated dsDNA breaks can be detected by cytometric analysis (40). Early studies showed the restricted binding of the anthracycline doxorubicin to DNA within chromatin compared to calf thymus free DNA and the ability of related drugs to induce compaction of isolated chromatin (58,59). Further evidence indicates that anthracycline antibiotic exposure can lead to chromatin unfolding and aggregation (60), DNA torsional changes (61) and histone eviction from open chromatin (62). Such drugs are also self-reporting probes

for cellular and nuclear micro-pharmacokinetics enabled by their intrinsic fluorescence tracked in real-time by flow cytometry (63,64).

Resolving chromatin organisation:

Super Resolution Optical Microscopy

New developments in fluorescent sensors based on small-molecule dyes or fluorescent proteins (13) are in parallel with expanding options for advanced microscopy methods for visualizing chromatin structure (4). Fluorescent sensors for monitoring proteins, DNA, RNA, small molecules and ions (e.g. Zn^{2+}) can all exploit super-resolution microscopy (13). Some of these approaches are noted briefly here. Super-resolution fluorescence microscopy encompasses multiple techniques (65) that are applicable to probing chromatin structure (4,66-70). Super-resolution microscopy effectively breaches the diffraction limit of optical microscopy and efforts continue to accrue techniques reaching signal resolution at length scales of sub-20 nm. Typical approaches employ the principles of patterned illumination light, such as Saturated Structured Illumination Microscopy (SIM) and Stimulated Emission Depletion (STED). Alternatively, some methods such as Stochastic Optical Reconstruction Microscopy (STORM), Photoactivation Localization Microscopy (PALM), and fluorescence-based fPALM allow for single molecule detection and localization applicable to the imaging of chromatin organisation (4). Approaches include the use of a fluorescent label to image DNA and chromatin *in situ* at the single-molecule level (71-73). A recent application of the DNA dye Vybrant®DyeCycle Violet with single molecule localization microscopy (SMLM) has generated images of DNA in nuclei of fixed mammalian cells (74). It is estimated that using fixed whole cells and standard DNA dyes, a structural resolution of chromatin is attainable of the order of 50-100 nm using SMLM (72). The use of Spectral Precision Distance/Position Determination Microscopy (SPDM) has the potential to reveal nuclear nanostructures down to few tens of nanometre resolution (75). Several intercalating and minor-groove binding DNA dyes can be used to register (optically isolate) a few DNA-binding dye signals at a time using a variation of Binding-Activated Localization Microscopy (BALM) (71). We should also remember that unmodified nucleic acids can show stochastic fluorescence switching at physiological concentrations under visible light illumination. This presents an opportunity for label-free super-resolution imaging of DNA directly (76). However, at this stage super-resolution approaches cannot address the problem of how to visualise and reconstruct chromatin ultrastructure through large 3D volumes of intact cells – a problem familiar in cytometry.

ChromEMT

The practical limits on imaging resolution restrict the ability to visualise chromatin organisation *in situ* for informative 3D volumes. A recent report has broken through this analysis barrier for intact cells using a new method. ChromEMT enables DNA and chromatin ultrastructure to be visualized and reconstructed unambiguously through large 3D volumes (77). The ChromEMT method combines electron microscopy tomography (EMT) with a labelling method that selectively enhances the contrast for DNA structures. The ChromEMT technique exploits unique properties of the fluorescent anthraquinone DNA dye DRAQ5, which upon photon-activation can catalyse the deposition of diaminobenzidine (DAB) polymers on the chromatin surface, enabling structures to be subsequently

visualized with OsO_4 in EM [Figure 2; (77)]. DAB photo-oxidation has been used in a range of studies that correlate light and electron microscopy, for nanoparticle location and the residence of low levels of photosensitizing molecules (78). Advances in multi-tilt EMT have allowed researchers to reveal the chromatin ultrastructure and 3D packing of DNA in both human interphase cells and mitotic chromosomes. Critically, it appears that there is a disordered nature of the chromatin chains revealed by ChromEMT. These chains are flexible, bending and folding into different packing densities. Dynamic changes in packing density provide opportunities to fine tune accessibility to DNA sequences, nucleosome variations and chromatin modifications. These variations in packing density will be important at both local and global levels within the intact nucleus. This opens a new level of organisation or indeed dis-organisation for exerting control over function. The anthraquinone dye used in ChromEMT recalls a lineage of molecules that link probe and drug properties employed in nuclear cytometry.

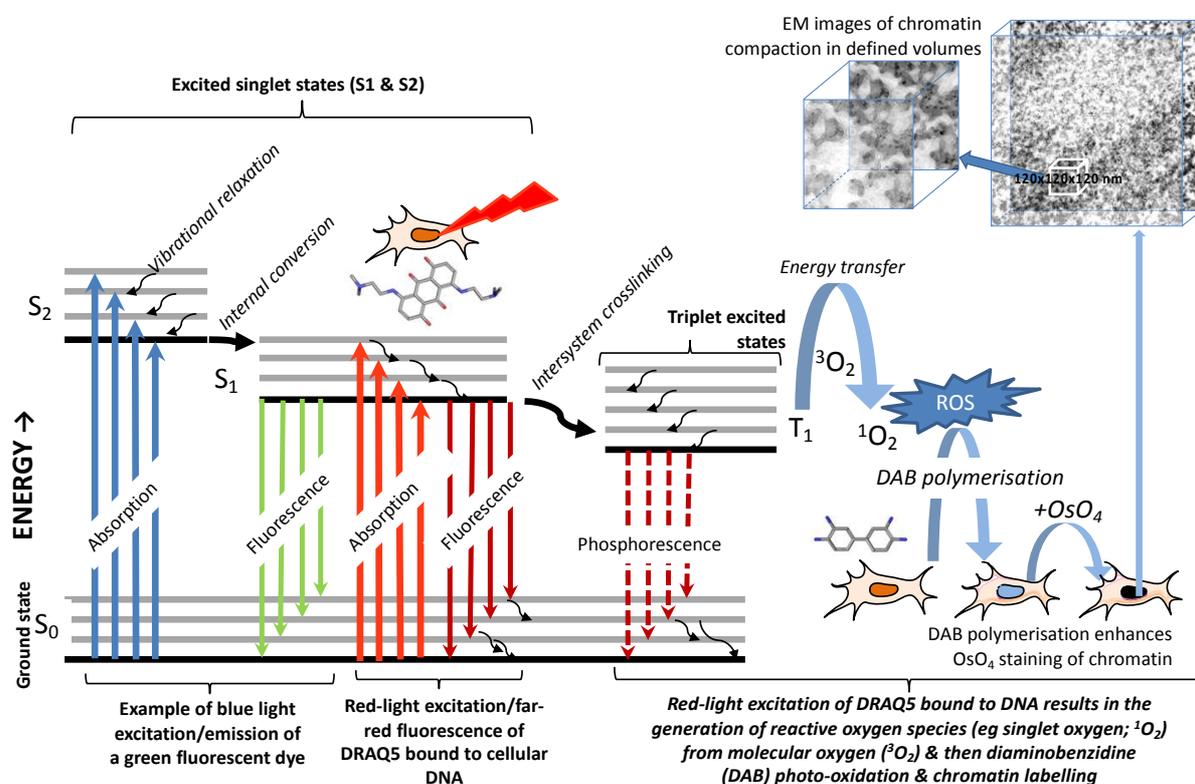


Figure 2. ChromEMT: Jablonski energy diagram of DRAQ5 excitation, fluorescence and the formation of a triplet excited state generating reactive oxygen species (ROS) that enable subsequent chromatin

visualisation at low scalar distances. Diagrammatic sequence shows the photo-oxidative catalysis of the deposition of osmiophilic DAB polymers on DNA in chromatin in situ (77).

Nuclear cytometry: DNA reporting

The measurement of cellular DNA content, a common laboratory procedure (12,79), has been dependent upon the stoichiometric reporting capacity of fluorescent nucleic acid-affinic dyes.

In terms of nuclear cytometry, several classes of 'vital' DNA fluorescence probes, in addition to the bis-benzimidazoles Hoechst™ dyes offer the ability to explore chromatin dynamics in live cells. This expanding palette of probes includes: Syto61™, DRAQ5™ and Vybrant® DyeCycle™. The interactions of a range of fluorescent dyes such as quinacrine, Hoechst 33258 (80), daunomycin, chromomycin A3 and 7-aminoactinomycin D with DNA offer multiple probe-based approaches for exploring chromatin structure (5). A recent addition to the suite of small-molecule fluorophores is the near-infrared (IR) silicon-rhodamine dyes with spectral properties that aid in vivo imaging (81). Spectral analysis can reveal modes of binding, sequence selectivity, probe interactions, consequences of fixation and selectivity of nuclease digestion. At a fundamental level, probe performance can be expected to obey the law of mass action in relation to unbound fluorochrome concentration (12). In studies on anticancer drugs there is always the possibility of interaction between the drug and the fluorochrome used to simultaneously probe DNA content. Since some of the DNA-binding drugs are fluorescent, their emission can directly overlap with that of the probe, through Förster resonance energy transfer, and affect the efficiency of probe detection (82).

With all probes there are caveats. Prolonged exposure of live cells to the nominally non-permeable dye propidium iodide (PI) reveals a background granular distribution of the probe in the cytoplasm, consistent with accumulation in endosomes, and also dye binding to nucleolar RNA in live cells (83). DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) is usually referred to as a semi membrane-permeant dye because of its reduced penetration through viable cell membranes but it is concentration sensitive with respect to toxicity especially at levels for attempts at DNA content reporting (84). A non-permeable very low toxicity dye, such as DRAQ7, offers the opportunity to monitor subpopulations of cells that lost cellular barriers to chromatin access but without perturbing the viable cell fraction (85). This approach is particularly useful when monitoring transitions in cell behaviour (86). In the case of the of vital cell dyes, problems of cell proliferation inhibition (14) and phototoxicity (87) can arise, but these effects will be time, dose and system dependent. The anthraquinone DRAQ5 when used at levels that reveal efficient DNA content reporting in live cells can interfere with the binding of H2B core histones to DNA, not observed after binding to DNA of a minor groove binder Syto17 (88,89). Hoechst 33342, DRAQ5, and DyeCycle Violet induce various degrees of DNA damage responses and cell cycle changes, which should be a matter of concern when using these dyes as supravital DNA probes inappropriately (90). Photo-toxicity of dyes is an issue. This is particularly the case a shorter wavelengths as observed with UV-excited Hoechst 33342 in time-lapse fluorescence microscopy (87). However, this known 'adverse' effect of UVA irradiation on dye-treated cells can be used to investigate the kinetics of dye residence at critical chromatin sites capable of generating photo-induced DNA-protein crosslinks (91). The potential for photosensitisation is

appreciated in the context of photothermal therapy (PTT) and photodynamic therapy (PDT). Here there have been recent advances in the synthesis and application of NIR-absorbing organic nanoparticles as phototherapeutic ‘nanoagents’ (92).

Small DNA-affinic molecules can be used to reveal not only DNA content but also accessibility within higher orders of chromatin structure. This opportunity was recognised in earlier cytometry studies (93) by exploiting the metachromatic properties of acridine orange (AO) (94,95). Many of our current methodological approaches and signal interpretations were developed using this cytometric probe. Differences in chromatin structure can be revealed by the metachromatic effects detecting DNA ‘melting’ – the differential susceptibility of DNA in situ to undergo denaturation upon exposure to heat or acid. Differential stainability of dsDNA (green fluorescence) *versus* ssDNA (red fluorescence) with AO made it possible to discriminate between G_0 , G_1 , S, G_2 and M cells (94,95) (Fig. 3). Multiple cytometry techniques have been applied to cell cycle profiling, including exploitation of chromatin-protein bindings events, chromatin modification and the nuclear translocation of cyclins. DNA content and chromatin-bound proteins to reveal sub-phases in G_1 (96). Mass cytometry using 5-iodo-2-deoxyuridine (IdU) can co-mark cells in S phase using cyclin antibodies and the phosphorylation patterns a specific histone H3 (97). Live cell tracking of a cyclin B1-GFP sensor can detect cell-cycle phase routes to mitotic traverse, arrest or endoreduplication confirmed by flow cytometric mapping using DRAQ5 (98).

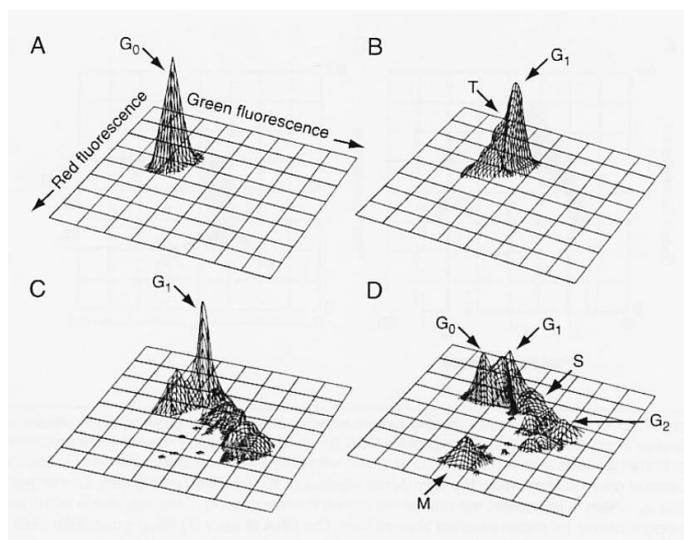


Fig. 3. Cytometric analysis showing differences in chromatin structure of lymphocytes in different phases of the cell cycle. Sub-sets revealed by changes in susceptibility of DNA to denaturation induced by acid followed by differential staining of dsDNA versus ssDNA with metachromatic fluorochrome acridine orange (AO). (A) Unstimulated cells, (B) cells stimulated with phytohemagglutinin (PHA) for 18 h, (C) cells stimulated for 3 days, (D) cells stimulated for 3 days; vinblastine included in the cultures for the final 6 h to arrest cells in mitosis. Evident is the transition (T) cells from G_0 to G_1 after 18 h, associated with an increase in green fluorescence and a decrease in red. Subpopulations of cells in G_0 , G_1 , S, G_2 and M can be distinguished based on changes in green and red fluorescence (94,95).

Fluorescent dye-chromatin interactions borrow from the experience with chromosome banding. These can reveal the spectacular capacity of cells to show shifts in chromosome organisation at its highest levels (99,100). At a highly practical level, metachromatic staining by AO reveals any defective packaging of DNA in chromatin of infertile sperm cells (101). Fig. 4 shows the classic flow cytometry methodological study of human fertile versus infertile sperm cells (102). Defective packaging of DNA correlates with DNA fragmentation, not unlike that seen in apoptotic cells (103,104). This methodology was adopted in clinical practice for detecting fertility status of human males and in animal husbandry (105,106).

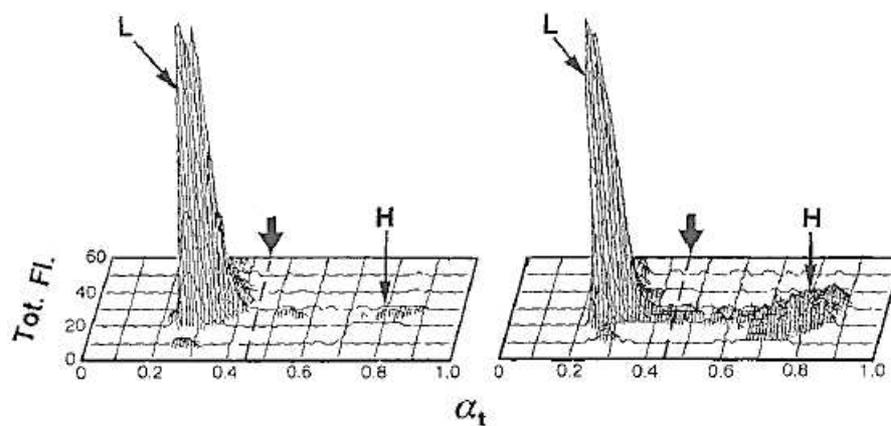


Figure 4. Susceptibility of DNA to denaturation as detected by staining with AO reveals the presence of human sperm cells with defective chromatin structure (H) correlated with infertility. *Bivariate frequency distribution histograms representing intensity of total fluorescence (Tot. Fl.; red+ green) versus α_t (ratio of red to total fluorescence intensity) of two samples of sperm cells, one characterized by a small fraction of cells with high sensitivity of DNA in situ to denaturation (left) and the other with a higher proportion of such cells (right). The borderline discriminating between the cells characterized by low (L) and high (H) sensitivity to denaturation (α_t , index) is marked by thick arrows. The frequency of H cells in infertile subjects was shown to be correlated with frequency of cells with fragmented DNA detected by the TUNEL assay (103).*

In the native nucleus, the extent and modes of binding of DNA interacting agents are informed not only by agent properties and chromatin access but also by cellular routes for handling small molecule probes. For example, contrast to fixed cells, live cells exposed to AO at low concentrations (<5 μ M) selectively accumulate the probe in acidic vesicles, including lysosomes where it luminesces red, and is not a useful chromatin probe (107,108). The situation is complicated further by dynamic chromatin structures responding to the presence of a probe/drug itself and linked downstream stress responses. Not surprisingly there is significant interest, in both nuclear cytometry and anticancer targeting strategies, to gain an understanding of critical chromatin structures and their perturbation.

Nuclear cytometry: alternatives

Not all methods depend upon the intrinsic or enhanced fluorescence of a probe or indeed that of a nuclear-locating drug. Mass cytometry has exploited metal-based unique signals for DNA-affinic molecules for: the study of cellular uptake and the linked anticancer effects of platinum-containing drugs (109), the use of cisplatin-antibody-conjugates for immunophenotyping (110) and the incorporation of a platinum-based covalent reagent to act as a discriminator in cell viability measurements (111). Alternative detection modalities can assess the subcellular disposition of reporter probes. Raman spectroscopy as applied to microscopy has been used to measure the chemical signature of a sample or identify the presence and quantity of a molecular species (112) (Figure 5). The characteristics and behaviour of a probe can in turn be used to extract biological information via the state of the probe at specific locations. The two typical methods of collecting the Raman spectral data to generate images are Raman mapping and Raman imaging. Raman mapping collects a spectral hypercube (a Raman spectrum from each position on the sample in a single file), rather than a simple intensity image. The hypercube is analysed to produce Raman images. There are several Raman mapping methods, including point-by-point mapping and line focus mapping. Here, there is a balance between under-sampling and acquisition time for sample regions of interest. Spatial resolution is determined by a combination of the laser spot size and the spacing between acquisition points on the sample (e.g. sample stage step sizes down to 100 nm). On the other hand, Raman imaging allows for rapid acquisition, by collecting spectral intensity values simultaneously from an entire region of interest, especially if high laser power is available. However this approach yields limited information with an ultimate resolution to a little under a micrometre.

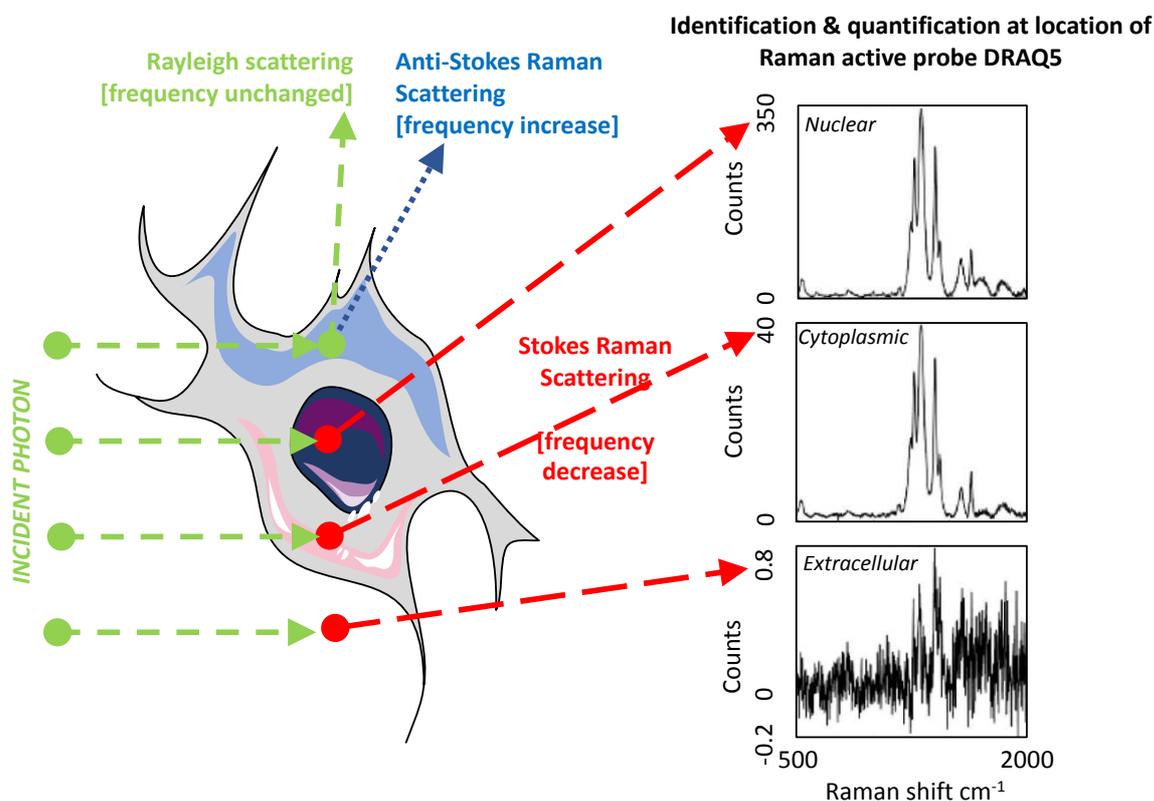


Figure 5: Diagrammatic representation of some of the light scattering modes of interest and the differential detection of a Raman signature for a cell permeant DNA binding probe. The more familiar Rayleigh scattering can be described as ‘elastic’ scattering since the photon energies of the scattered photons are not changed. When a Raman active probe is irradiated with photons of a selected frequency, that avoids peak fluorescence excitation, a minority of the incident photons interact with a vibrational mode of the irradiated probe and are ‘inelastically’ scattered. The inelastically scattered photons are shifted in frequency. This shift can be to a higher frequency (anti-Stokes) or to a lower frequency (Stokes). The frequency versus intensity Raman spectrum provides a unique detection signal for the probe at a selected location. (Acknowledgement: exemplar data for DRAQ5 provided courtesy of L Jamieson, D Graham & K Faulds, Centre for Molecular Nanometrology, Department of Pure and Applied Chemistry, University of Strathclyde, UK).

Many materials have characteristic Raman spectra, with a growing number of applications within biology. The approach can provide chemical and compositional information and does not typically suffer from interference from water molecules. Further, cellular components, including DNA, have distinct intrinsic Raman scattering spectra (113). Chromatin density variation among the individual sub-phases of mitosis affect Raman and infrared micro-spectroscopic intensities (114). Label-free DNA imaging in vivo has been demonstrated using stimulated Raman scattering microscopy (115,116). Hyperspectral Coherent anti-Stokes Raman scattering (CARS) microscopy (117) can be used to provide label-free quantitative volumetric imaging of cell composition defined in terms of water, protein, chromatin and lipids (Fig. 6).

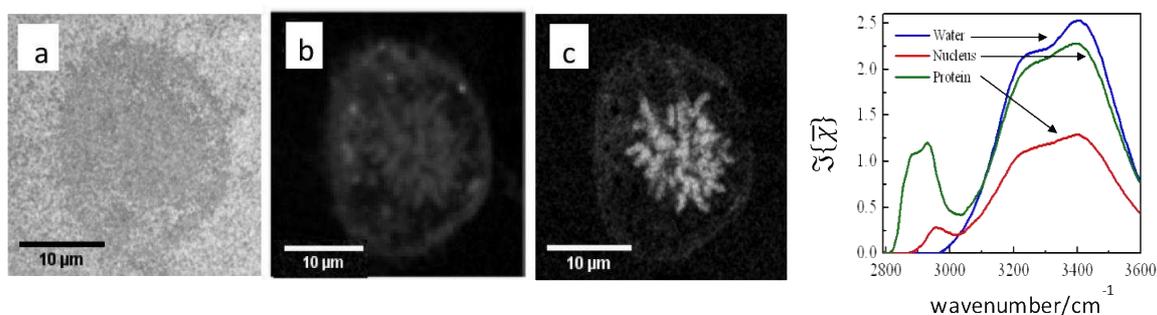


Figure 6 Hyperspectral CARS of a mitotic cell - detection of key cellular components and their spectra in a human osteosarcoma cell. Cells were imaged in 3D in the CH stretching vibrational range ($\omega=2400-3800\text{ cm}^{-1}$) using a 60X 1.27NA water objective and 1.4NA oil condenser. The point spread function (PSF) informs the imaged volume for chemical components [volume dimensions of approximately $0.3\ \mu\text{m}$ lateral and $0.9\ \mu\text{m}$ axial full width at half maximum (FWHM)]. Note that the imaged volume contains about 95% water. Y axis shows hyperspectral averaged $\chi''(\chi)$ data (118). To analyze the data, a three step method was applied which includes singular value decomposition (SVD), phase corrected Kramer's Kronig (PCKK)(118) and factorization into susceptibilities and concentrations of chemical components (FSC3) [(a: blue = water; b: green = protein; c: red = DNA)]. (Unpublished: exemplar data by A Karuna, F Masia, P Borri, R Errington, and W Langbein at the Schools of Physics, Biosciences and Medicine, Cardiff University, UK).

Mass Spectrometry Imaging (MSI) can qualitatively describe drug distribution in 2D tissue sections while 3D MSI can reveal the heterogeneity in tumours (119). MALDI imaging mass spectrometry has been applied to the analysis of the spatial distribution of histone modifications in tissues (120), with recent studies achieving high lateral resolution of the order of 5 micrometre approaching a cell-to-cell methodology. Partial-wave spectroscopic (PWS) microscopy is a label-free quantitative imaging technique (resolution range of 20 and 200 nm), capable of tracking changes in chromatin structure after DNA damage in live cells (121). This approach reveals that UV-excited Hoechst 33342 can cause damage to chromatin within seconds. Over a time-frame of minutes, the dye itself can cause a global alteration in chromatin nano-architecture independent of its excitation (121). PWS microscopy reveals the not unexpected chromatin disrupting effects of fixation (122). This disruption highlights the relevance of live cell imaging techniques, or the advantage of cryo-fixation, when exploring native chromatin organisation (122). The ability to combine PWS microscopy with fluorescence confocal microscopy for the co-localization of cellular nanostructures is a welcome route for functional annotation (123). Alternative modes of light interaction with DNA dyes can be exploited in nuclear cytometry. For example, the unique light absorption properties and high DNA affinity DRAQ5 (124) coupled with low fluorescence quantum yield (125), have been exploited in hybrid ultra-high frequency acoustic/photoacoustic microscopy. The generation of photoacoustic signals from a cell's nuclei reveals gross conformation and nuclear dimensions (126).

Apoptosis, autophagy & NETs

Chromatin disassembly is a feature of the various cell death processes and will only be discussed here briefly. Multiple cytometric methods can track the irreversible apoptotic process of chromatin disassembly (127,128) and real time progression to the '*fait accompli*' of plasma membrane disruption (85). In the case of apoptosis, nuclear DNA undergoes extensive fragmentation and release from the cell within apoptotic bodies (42). RNA and DNA are segregated and packed into separate apoptotic bodies (129) detectable in peripheral blood (130). Specialization of phagosomes of macrophages facilitates heterophagic degradation of nucleic acids during apoptosis (129). An exciting and emerging area is the balance between autophagy, providing an environment for cell survival, versus the enactment of cell death processes via apoptosis. The cell survival and housekeeping process of autophagy involves the breaking down and reusing of cytoplasm components. Fluorescence microscopy and flow cytometry is commonly used to study autophagy of discrete structures such as mitochondria (131) and lysosomes (107). Flow cytometric methods are also available to detect the linked changes in organelle mass using MitoTracker Green (MTG) and Endoplasmic Reticulum Tracker Green (ERTG) (132). Evidence is mounting that there is cross-talk between the dysfunction of apoptotic and autophagic pathways in disease states including neurodegeneration (133) and cardiac pathology (134). Participants in this cross-talk are modifications to histones, providing critical structural codes (135).

Chromatin 'function' or rather 'value' can extend beyond the cell's boundary (136) presenting a distinct focus area for cytometry. The cell death process termed NETosis, creates homogenised

chromatin together with granule proteins that undergo release from actively disintegrating neutrophil granulocytes. A variety of triggers promote NET formation. These chromatin structures form a tethered extracellular mesh (Neutrophil Extracellular Traps; NETs; (137)). NETs enmesh and degrade virulence factors and kill bacteria. The DNA backbone of NETs is vulnerable to extracellular DNase digestion. Although NETosis is distinct from apoptosis and necrosis, there is conflicting evidence of its dependence on autophagy for extracellular DNA trap formation (138,139). Further there is a distinction between the formation of NETs from mitochondrial or nuclear DNA (140). Nuclear chromatin-originating DNA masses are less structured (the DNA mass being some 3-5-fold greater in volume than condensed chromatin) and less beneficial for antimicrobial trapping (141). The more structured mitochondrial originating NETs are built around the tight packaging of mtDNA as nucleoids and their organization into higher-ordered assemblies (142).

Developing cytometric methods that distinguish different NET origins and structures have applications in measuring inflammatory vascular injury and tissue damage (143) and in a variety of disease processes including autoimmunity, thrombosis, cancer and antiviral responses (144). NET formation has been examined using correlative microscopy - combining TEM, SEM, immunofluorescence and live cell imaging techniques (145). Such imaging approaches can be supported by automated image quantification software (146). A range of probe-based methods are also available for quantifying NETosis (145,147). They include flow cytometry and staining with the plasma membrane-impermeable DNA-binding dye SYTOX Green and its correlation with image-based detection (148). Co-staining for DNA (DAPI) and myeloperoxidase positivity has also been used (149). DRAQ5 and SYTOX Green staining patterns can enable NET detection by both flow cytometry (150) and imaging (151). DRAQ5 and human neutrophil elastase immunostaining and microscopy have been used to profile NETs capturing *Candida albicans* yeast cells (152). An interesting recent approach uses the ImageStream® platform (Millipore Sigma, Darmstadt, Germany) detecting the morphology of DRAQ5-stained NET DNA trails tethered to the remaining cell structure (153).

One man's probe is another man's poison

In vivo probes and drugs have to deal with adverse extracellular microenvironments (154) and a metabolic environment frequently determined by gene expression patterns (e.g. p450 family; (155)). These states can enhance or even frustrate delivery to targets (154). For example cytometry has revealed the DNA targeting potential of fluorescent anthraquinone-based prodrugs (156,157) in their response to their metabolic activation in hypoxic microenvironments (158). Flow- and image-assisted cytometry can profile individual molecular events in DNA damage responses (159) linked to effective targeting of a probe (160) or cross-resistance to a drug (161). The ability of drug molecules to quench Hoechst 33342-DNA fluorescence signatures can be used to track the binding of molecules with low fluorescence [e.g. the anti-leukemic drug 4'-(9-acridinylamino) methane sulfon-m-anisidide; m-AMSA; (162)] or spectrally distinct fluorescence properties (125,163). DNA targeting agents face cellular membrane barriers (112,164), active efflux (161) and sequestration at cytoplasmic sites (156). The nucleic acid target is clearly in competition with different kinds of molecules. Even the packaging of the genome by histone proteins into nucleosomes can alter how different DNA sites interact preferentially with an agent. A DNA affinic drug such as mitoxantrone (MTX) can significantly impact

upon chromatin protein residence and function (51,165,166), DNA topoisomerase function (167). The pharmacodynamic effects of MTX appear to be dependent upon persistence at the chromatin target (54) an indicator that probe residence time at target is critical. Probe efflux provides information on cell heterogeneity that can correlate with drug response heterogeneity. The detection of 'side-populations' characterizing stem cells (168) is based on the extent of the binding-dependent shift in the Hoechst 33342-DNA emission spectrum (169,170). Changes in Hoechst 33342 (171,172) and Vybrant® DyeCycle™ Violet (173) cellular fluorescence effectively track dye efflux capacity. The efflux signature of DNA interactive agents and drugs can be explored using a compartmental modelling approach, providing a mathematical description of the activity of the anti-cancer agent. Such models can take into account intracellular modification and delivery to a nuclear DNA target (27). This can also be extended to model the subsequent perturbation of the cell cycle (174). Smart DNA-targeted reporters will enable mathematical constructs of single cells that link Micro-Pharmacokinetics and cellular Pharmacodynamics with specific Cellular Descriptors (μ PK-PD-CD). Descriptors, such as aldehyde dehydrogenase enzyme expression (175), bring a finer cytometric dimension to early drug development (176).

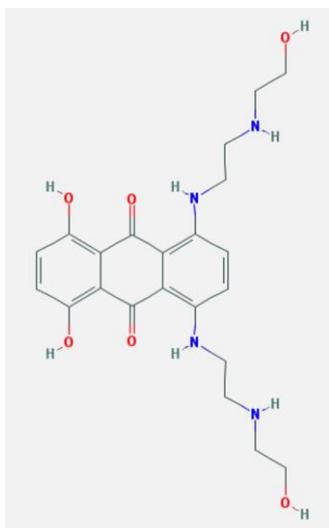


Figure 7: Chemical structure of Mitoxantrone (MTX) - an anthracenedione antibiotic with antineoplastic activity. Chemical structure information and properties are obtainable from the PubChem Substance and Compound databases (177).

The problem of understanding the biological impact of a DNA targeting molecule and probe properties in the context of chromatin structure is exemplified here by the anthracenediones. Mitoxantrone (MTX; Novantrone®), (1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione) is an anthracenedione - structurally related to the classical anthracyclines. MTX has also provided an impetus for the generation of derivatives including prodrug forms (178-181) for a more selective targeting of tumour cell populations (182). Its behaviour in the chromatin environment (166) is an exemplar of multi-level action (40). A principal mechanism of action of MTX is the persistent inhibition of DNA topoisomerase complexes (54). However, MTX also has a striking ability to condense nucleic acids by both trapping and excluding chromatin proteins (166,183). The MTX chromophore ($Ex\lambda_{max}$ 610 and 660 nm; $Em\lambda_{max}$ 685 nm ; (184)) permits fluorescence microscopy and flow cytometry to determine uptake and nuclear distribution revealing drug resistance (185) and the behaviour of alkylamino-anthraquinones derivatives and their N-oxides (156). The structurally related far-red fluorescent DNA dyes and the nuclear counterstains DRAQ5™ (124,186-188), DRAQ7™

(85,128,189-191) and the spectrally shifted CyTRAK Orange™ (192) have applications in nuclear cytometry (193). The spectrally-compatible combination of the nuclear stain DRAQ5 and the anionic counterstain eosin also provides a dual-component fluorescent staining protocol. This is analogous to haematoxylin & eosin and intended for use on fresh, non-sectioned tissues (186) in clinical settings – an innovation loop in nuclear cytometry that now spans centuries (194).

Conclusion

In terms of nuclear cytometry there is a widening of the opportunities to deploy smart molecular DNA probes, not restricted by their fluorescence profiles. Novel probes offer distinct and often unique properties. Molecular probe design is clearly informed by drug development highlighting a constant caveat for the use of vital probes, in which their biological effects can be a known unknown. The ambition here is to reveal fundamental aspects of nuclear function and chromatin form. New opportunities arise from understanding the photophysical and photochemical properties of probes in conjunction with the targeting of discrete biological structures while also exploiting new detection modalities. Downstream impact lies in the promise of methodologies that can inform chromatin structures and organisational levels that are targets for anticancer drugs or indeed reflect disease progression. Further, using these new approaches to nuclear cytometry, to describe the chromatin changes that occur both globally and locally during key transitions such as a somatic to pluripotent states (195,196), will reveal the extent to which such changes have regulatory roles in both disease and health.

Declaration & Acknowledgements

Authors PJS and RJE declare that they are non-executive directors of Biostatus Ltd, the commercial supplier of DRAQ5, DRAQ7 and CyTRAK Orange. ZD has no conflicts to declare. The authors note: Vybrant® DyeCycle™ Ruby stain Syto61™ are trademarks of Thermo Fisher Scientific USA (Molecular Probes Inc.); MitoTracker™ and ER-Tracker™ are registered trademarks of Molecular Probes, Inc.; DRAQ5™, DRAQ7™ and CyTRAK™ are trademarks of Biostatus Ltd; Hoechst™ is a trademark of Sanofi

USA; [®]Adriamycin is a registered trademark of Pharmacia & Upjohn S.P.A.; Novantrone is a trademark American Cyanamid Company. Chemical structures are obtainable from the PubChem Substance and Compound databases (177). ZD is supported by The Robert A. Welke Cancer Research Foundation.

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