Phenotypic Screening and in vitro Toxicology: applications of far-red fluorescing DNA-specific anthraquinones in cell-based assays.

The supravital DNA dye DRAQ5 (Smith, 2000) has been widely applied to cell-based assays for biological and medical research. Being cell permeant and conveniently water-soluble it can be applied to live or fixed cells and tissues to demark the nucleus of a cell. At its most basic this permits the “gating” of nucleated from enucleated cells, without the need for red blood cell (RBC) lysis, thus limiting the need for time-consuming and often perturbing sample preparation. Its far-red emission means it can be combined with the majority of antibody fluorescence tags to allow up to 5 or 6 colour analysis in 2-laser flow cytometry. This has been demonstrated in blood and bone marrow for multiparameter leucocyte differential analysis in both human (Björnsson et al, 2008) and rats (Kurata et al, 2007), in the latter case to track hematotoxicity. Similar gating strategies have been demonstrated with the related supravital DNA dye CyTRAK Orange (Errington et al, 2006), which is compatible with red-excited fluors (Dimmick, 2008). Importantly, both DRAQ5 and CyTRAK Orange are spectrally compatible with GFP and Alexafluor 488/FITC.

Additionally, in flow cytometry, DRAQ5 shows excellent stoichiometry with DNA content in live (or fixed cells) which has led to its wide adoption for high resolution cell cycle determinations in haemat-o-oncology applications, such as ploidy status, S-phase analysis - indicative of proliferation and minimal residual disease detection (Plander et al, 2003, and others). This has also made DRAQ5 highly suitable for use in plate-based cytometry (Payne Et al, 2007) and in-cell western assays, where again the DNA dye can be used as an event “trigger” and the total signal is directly proportional to the number of cells present in a single well and can thus be used to normalise the analyte signal for well-to-well cell number variances (Hannoush, 2008). Further, in high content imaging assays DNA content profiles have been demonstrated with DRAQ5, to study chemical perturbation of the cell cycle (May et al, 2008).

The particular spectral and chemical features of DRAQ5 and CyTRAK Orange mean they are compatible with aqueous media and fixatives. Conveniently, they are supplied in aqueous solution and can be applied as the final step in a sample preparation.

In immunofluorescence microscopy these Fluorescein-/GFP-compatible DNA dyes have become standard reagents as nuclear counterstains cited in numerous publications since commercial release in 2001, as shown opposite combined with AlexaFluor 488-tagged anti-β-tubulin on U2-OS cells. An extension of this, the advent of cell-based assays for drug discovery has seen the wide adoption of DRAQ5 and CyTRAK Orange as counterstains for cellular object identification. Since many assays use GFP-tagging as the readout, the lack of spectral overlap means that these dyes always permit single-pass scanning for the two fluorescent signals, thereby halving microtiter plate scanning times.
One remarkable feature of both dyes is the presence of a weak cytoplasmic stain that can be used to more reliably segment nuclear and cytoplasmic compartments of cells, by establishing a threshold on nuclear intensity, creating a mask for this and then a second threshold for the signal outside this, equivalent to the cytoplasmic region. Thereby, two compartments are reliably segmented with the use of only one fluorescent channel (Haasen et al, 2006 and Loechel et al, 2006).

In the context of early in vitro toxicity alerts these parameters can be informative even in primary high content assay screens. In one study using DRAQ5 in a phenotypic screen for compounds modulating the prenylation pathway (Simonen et al, 2008) two simple readouts were beneficial and independent of each other: namely total cell numbers (nuclear count) as an indication of gross toxicity and increased brightness of the nuclear DRAQ5 signal in individual cells, indicative of nuclear condensation – one feature of toxicity. Extending this rationale further, in screens for inhibitors of HCV (Berke at al, 2010) and HIV (Gustin et al, 2009) cell infection where a wide panel of morphometric features were analysed for their ability to determine unwanted compound toxicity (such as shown in the simplified figure opposite).

This strategy is now employed across the industry, indicative of likely toxicity as early as a primary cell-based assay screen, at no additional assay cost other than the mining of imaging data and measurements therein. Subsequently, a number of dedicated cell health and toxicity assays have been developed taking advantage of the properties of DRAQ5. Notably, using DRAQ5 in panel with three further functional probes for mitochondrial membrane permeabilization, glutathione and reactive oxygen species (Xu et al., 2008) it was possible to robustly report cytotoxicity of compounds, in a four-colour cell-based imaging assay. The results correlated to a large panel of compounds with historically well-described and classified hepatotoxicity ranging in severity and the point of their removal from the development process or the market. One fascinating revelation of the work was the observation that the DNA probe DRAQ5™ exhibited a useful labelling of peri-nuclear lipid vesicles, indicative of drug-induced phospholipidosis in hepatocytes. Although this might not be surprising since a live cell-permeant probe might be expected to be lipophilic in nature, such a phenomenon has not been observed with other cell permeant DNA probe dyes and this remains an important feature of hepatotoxicity.

Similarly, as would be predicted, the small yet equally bright micronuclei resulting from genotoxicity can be detected proximal to the nucleus, both being DRAQ5™ labelled (Grieshaber et al., 2006). Interestingly however, one challenge for pharmaceutical development is the need to reliably automate this essential, rare-event detection assay. A recent paper (Westerink et al, 2011) used a novel combination of DNA dyes, Hoechst 33342 and DRAQ5™, to permit this. In essence (as shown in the cartoon opposite), proximally-located nuclei detected with Hoechst (A), their presence in a single...
cytoplasmic envelope confirmed by DRAQ5™ stain (B) and any small dsDNA event detected (C) with Hoechst 33342 following the two-feature combination would be reliably determined as a true micronucleus resulting from the genotoxic effects of a compound.

In **flow cytometry** and **fluorescence microscopy** (including high content screening), a reliable estimation of cell viability is important because it is central to assays for **apoptosis** and **in vitro toxicology**. Likewise, it is often a useful measure for sample quality and for robust phenotypic analysis of clinical samples. To better report cell viability DRAQ7™ (Edward, 2011) was developed from DRAQ5™. Essentially, this water-soluble probe has identical spectral properties to DRAQ5™ and as such does not overlap with the majority of visible range fluoros. Likewise, RNA binding is very weak (undetectable by flow cytometry). However, the chemical modification has rendered DRAQ7™ membrane impermeant, thus it does not cross the membrane of viable cells but rapidly enters “leaky” cells and labels nuclear DNA. DRAQ7™ is therefore a useful marker of cell membrane permeabilization, apoptosis, necrosis and dead cells. SU-DHL-4 cells cultured in the presence of DRAQ7™ at both the standard concentration of 3 µM or 3.3X excess show no significant impact on growth curves compared to untreated controls making DRAQ7™ an ideal candidate as a reporter of cell death in real-time, long-term viability and toxicity assays, as shown opposite. DRAQ7™ can be used as a new far-red reporter of cell viability and conversely cell membrane-permeabilization resulting from apoptosis and necrosis. As such, DRAQ7™ is an ideal spectrally-shifted replacement for agents such as DAPI, propidium iodide (PI) and TOTO-3 and may offer some new information and a wider assay window. For HCS studies in drug discovery and in vitro toxicology DRAQ7™ can be applied as a reporter of cell-membrane permeabilization, combined with live cell-permeant DNA dyes (e.g. CyTRAK Orange™ and Hoechst dyes). DRAQ7™ can be applied as a viability reporter in mitotic index assays and in studies into cell health in response to insults. As an example, for the typical HCS cell health assay, which combines a dye for “all events” (Hoechst 33342), a mitochondrial membrane potential reporter (e.g. TMRM) and a cell viability dye (TOTO-3), the latter component can be replaced with DRAQ7™ for less spectral overlap and thus wider detection windows for the TMRM and cell viability components whilst significantly reducing total assay reagent costs.

DRAQ7™ has been robustly tested and exemplified in key cytometry publications (Akagi et al, 2013a; Akagi et al, 2013b; Włodkowic et al, 2013) for compatibility with multi-colour experiments and uniquely for long-term, real-time analysis. It has recently been utilised in imaging procedures to monitor cell viability in 2-D and 3-D spheroid/micro-tissue assays including a study on glioblastoma-derived stem cell lines in response to a library of chemotherapeutic agents (detailed in a separate BioStatus/Imagen-Biotech white paper). Additionally, DRAQ7™ has been applied to a variety of **long-term and real-time cell health assays** that benefit from its fundamental features. For example, DRAQ7™ has been applied to monitoring cell health in 7-day culture of pancreas tissue (Marciniak et al, 2013), for real-time nanoparticle toxicity monitoring (Ware et al, 2014), for real-time study of mitochondrially-regulated apoptosis (Liang et al, 2015) and detecting subcutaneous extracellular dsDNA in better understanding its adjuvant role in vaccination (Wang et al, 2015).
Due to their unique chemical and spectral properties Supravital dyes DRAQ5™ and CyTRAK Orange™ and the viability dye DRAQ7™ offer new dimensions and opportunities for performance of high value phenotypic and in vitro toxicity cell-based assays in drug discovery and development, that can be applied across different platforms including flow cytometry, fluorescence microscopy and high content imaging platforms.

Reference List


BioStatus is very grateful to Imagen-Biotech for sharing this research data. For further details on their work visit:
www.imagen-biotech.com

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